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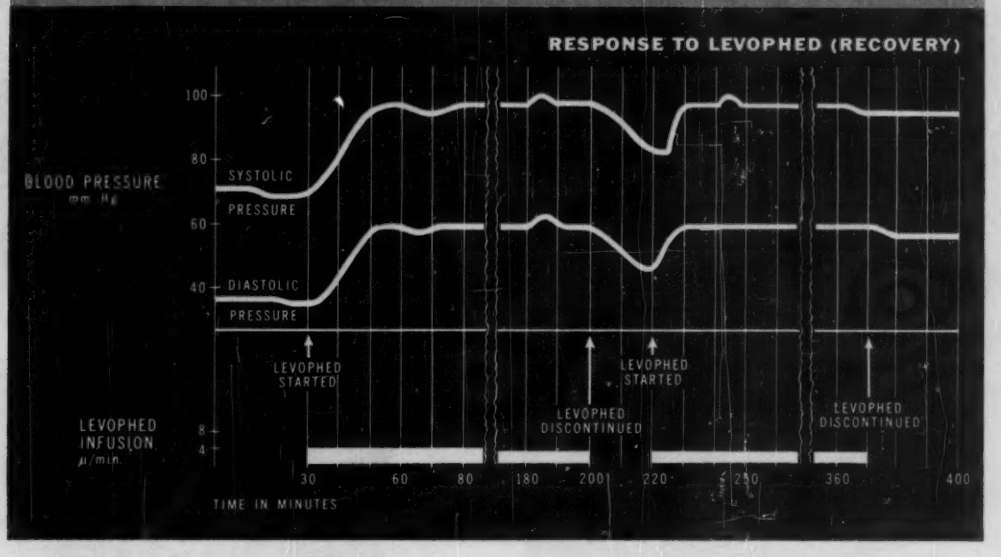

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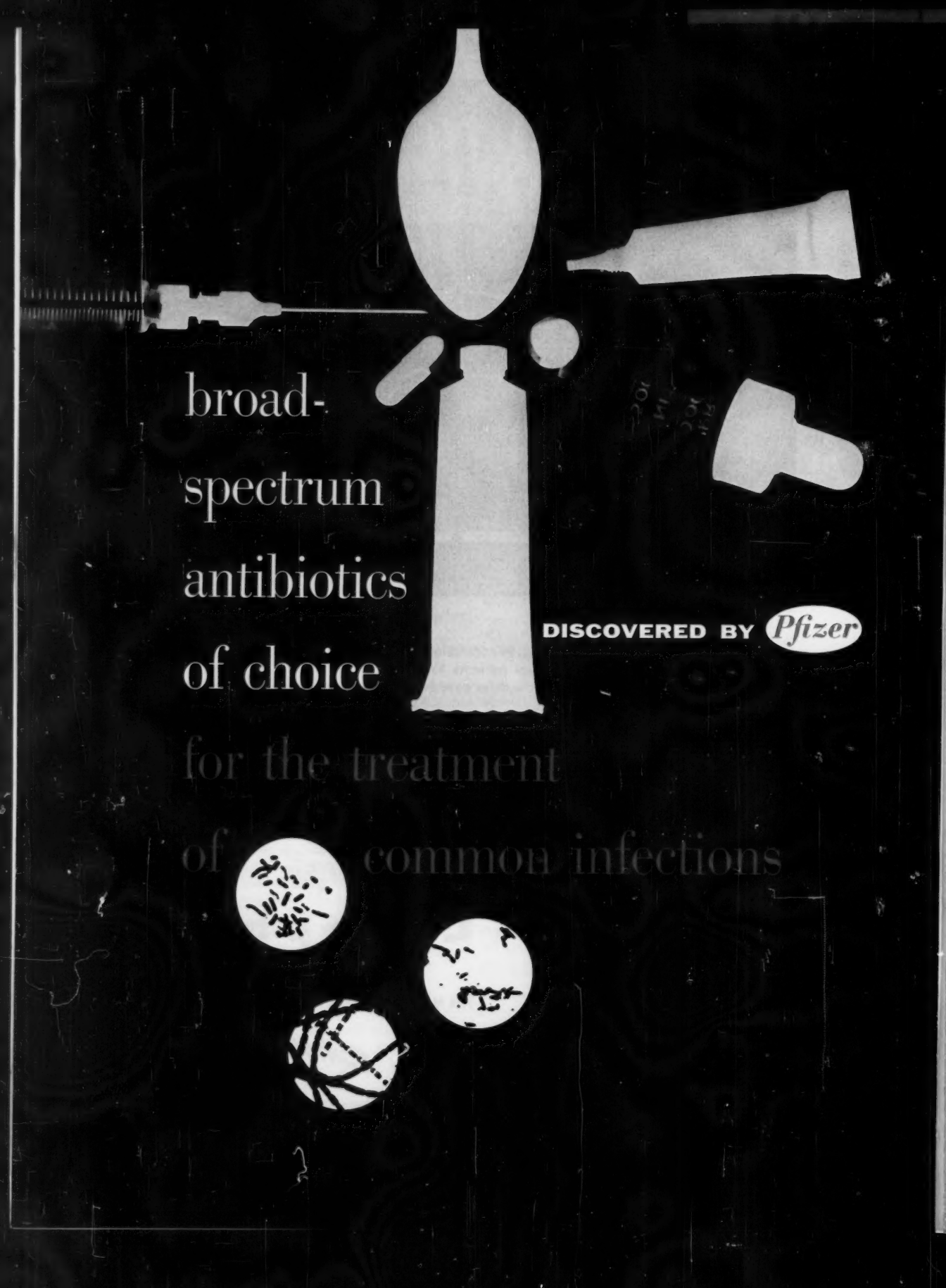
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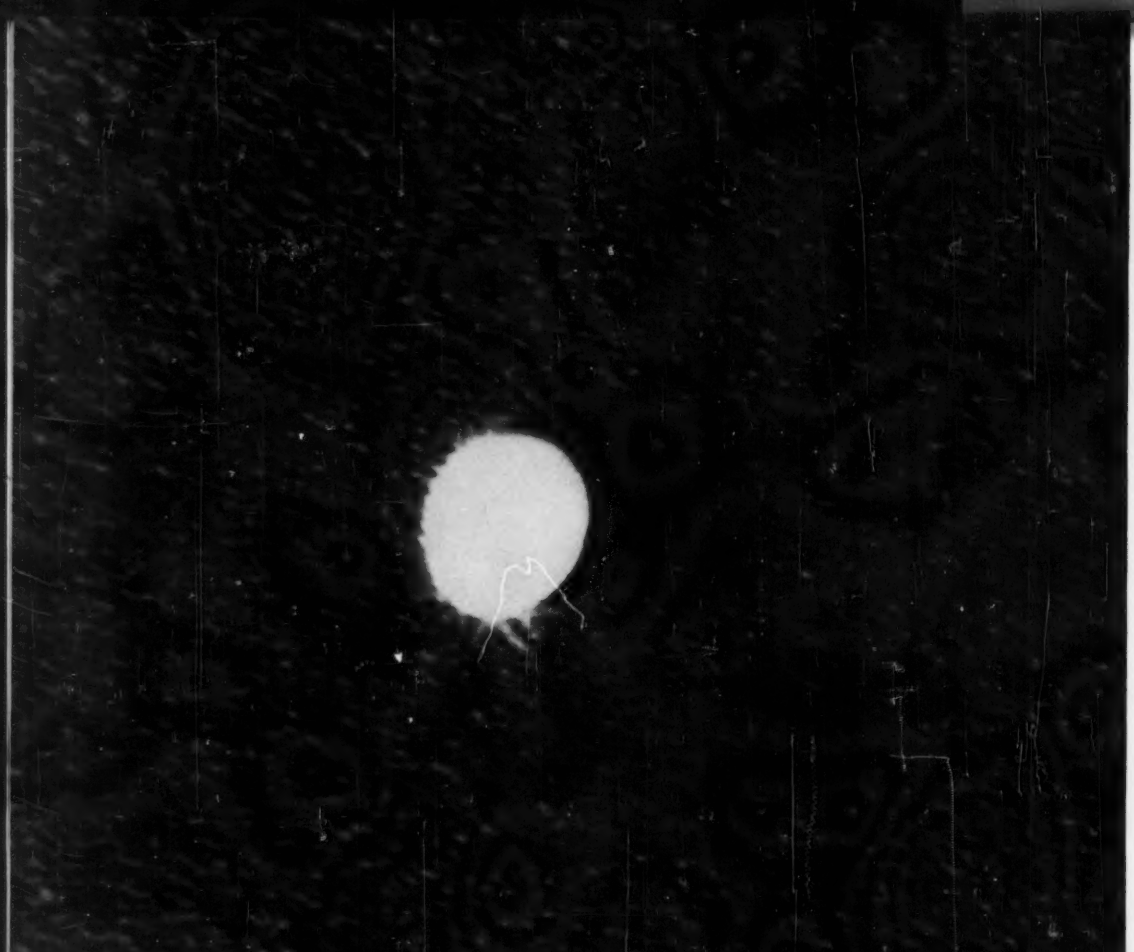

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*J. S. LaDue et al., *Science* 120, 497 (1954)

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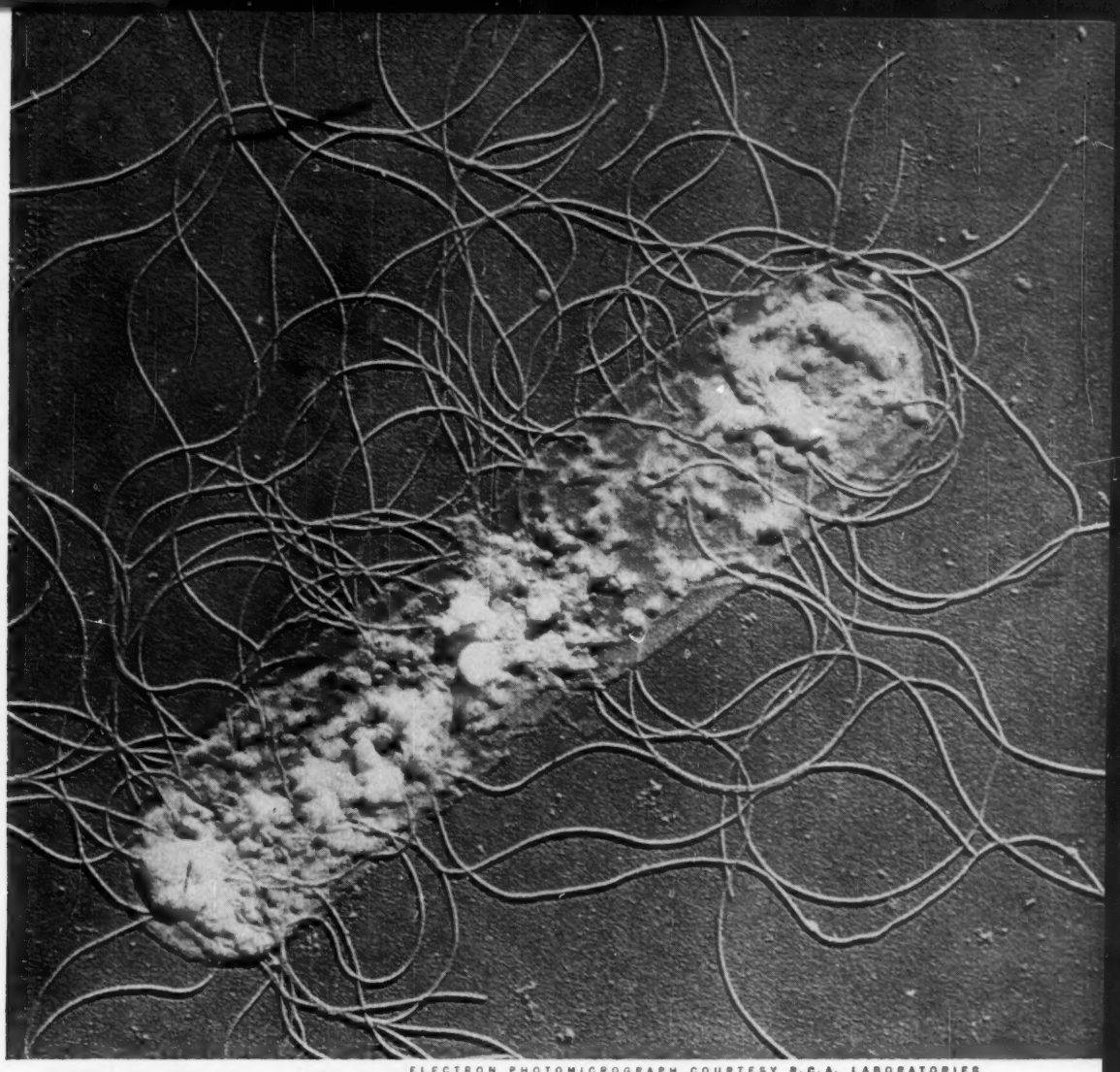

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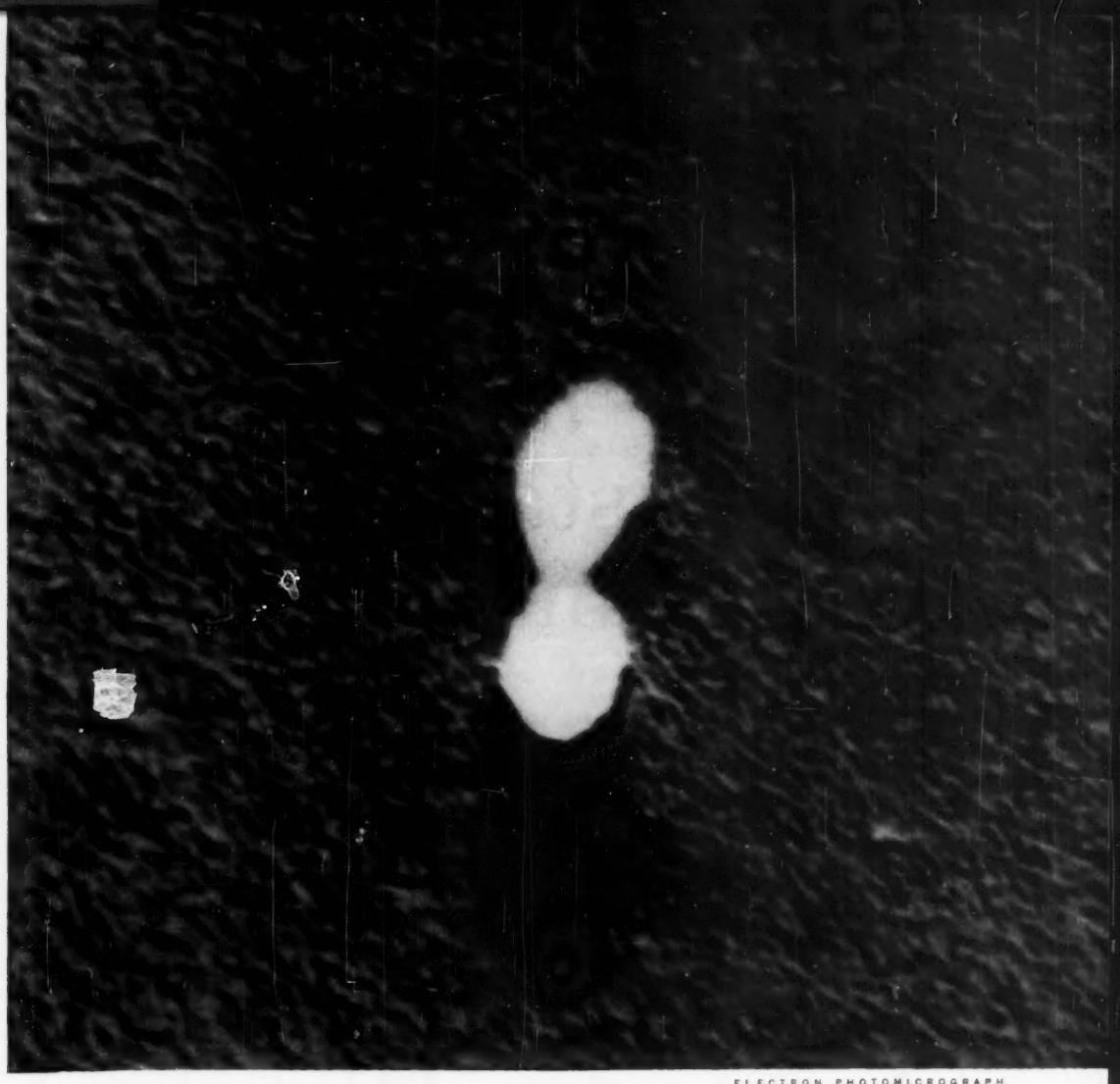

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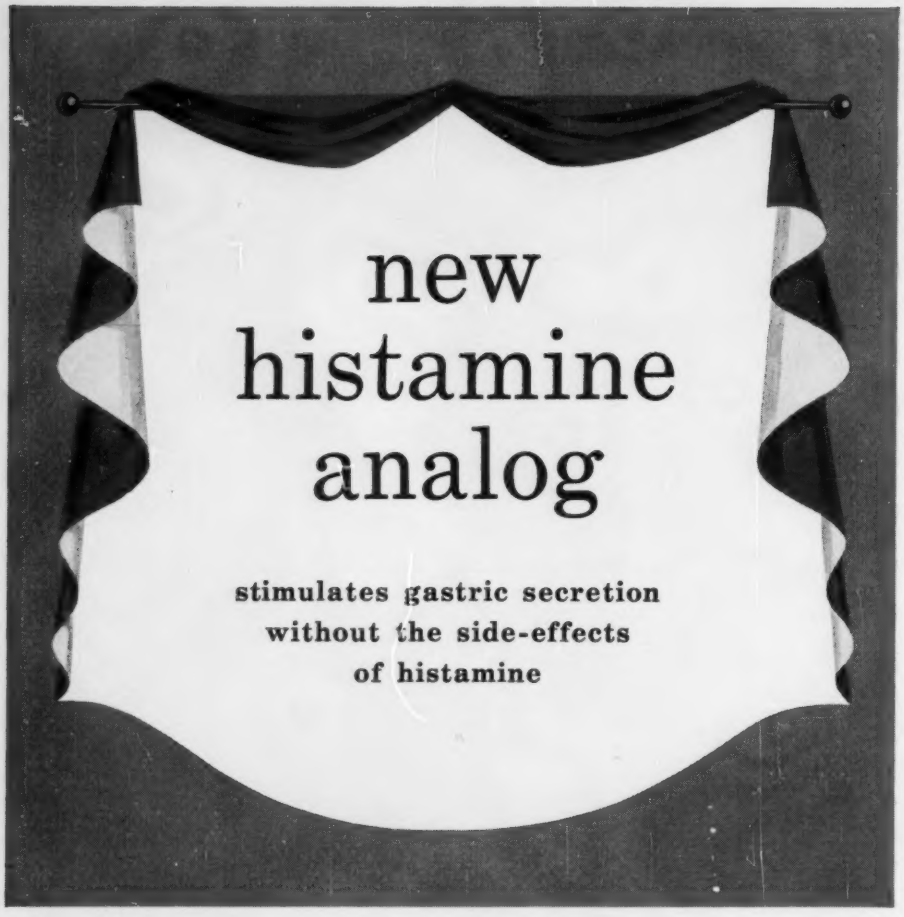

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TRANSCAPILLARY MIGRATION OF HEAVY WATER AND THIOCYANATE ION IN THE PULMONARY CIRCULATION OF NORMAL SUBJECTS AND PATIENTS WITH CONGESTIVE HEART FAILURE [1, 2, 3]

By LAWRENCE S. LILIENFIELD, EDWARD D. FREIS, EDWARD A. PARTENOPE, AND HAROLD J. MOROWITZ [4]

(From the Cardiovascular Research Laboratory, Georgetown University Medical Center and the Veterans Administration Hospital, Washington, D. C.)

(Submitted for publication August 5, 1954; accepted September 8, 1954)

During recent years numerous investigations have been concerned with the rates of transfer or exchange of various substances across capillary beds (1–6). These studies, by analyzing curves of disappearance over several minutes from arterial plasma of intravenously injected tracer substances, have provided some information relative to all capillary beds combined. However, this type of experiment has not permitted a close analysis of the behavior of a substance during its first passage through a single organ capillary bed.

Recently developed techniques (7–9) now permit the clinical investigator to examine the phenomena of transcapillary exchange of water and other diffusible substances in local vascular areas. This study was undertaken in an attempt to determine *in vivo* the diffusion characteristics of the pulmonary capillary bed in man with regard to deuterium oxide and an electrolyte, thiocyanate ion.

Seven normal subjects and seven patients with pulmonary congestion due to heart failure were studied. The resulting data relating to the pulmonary capillaries were then compared to those previously obtained using similar tracer substances in the capillaries of the human forearm (7).

[1] Presented in part at the annual meeting of the American Federation for Clinical Research, Atlantic City, New Jersey, May 2, 1954.

[2] Supported in part by the Squibb Institute for Medical Research, New Brunswick, New Jersey and the Irwin Neisler Co., Decatur, Illinois.

[3] Sponsored in part by the Veterans Administration. Published with approval of the Chief Medical Director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

[4] Biophysicist, National Heart Institute, National Institutes of Health, Bethesda, Maryland.

METHODS

A mixture of a non-diffusible substance (T-1824 or Evans blue dye) and substances whose diffusion is to be studied (deuterium oxide and thiocyanate ion) is injected into an afferent vessel of the organ under study. By sampling continuously from an efferent vessel for the period of the first circulation, the transcapillary exchange pattern can be determined.

The method is based on the principle that the relative concentrations of a mixture of diffusible and non-diffusible substances will be changed after passage through a capillary bed. The concentration of the non-diffusible substances in the effluent blood provides an index of the concentration of diffusible substance to be expected if none of the latter had been lost through the capillary walls. The difference between this expected concentration of the diffusible substance and that experimentally determined is a measure of the transcapillary loss (7).

A mixture usually consisting of 10 cc. of deuterium oxide, 10 cc. of 5 per cent sodium thiocyanate and 7 cc. of 0.5 per cent T-1824 dye was mixed well under sterile precautions. Approximately 20 cc. of this mixture was then injected rapidly from a calibrated syringe through an eighteen gauge needle usually into a basilic vein, although in four cases the injection was made through a cardiac catheter into the main pulmonary artery. Samples of blood were collected from the femoral artery through a fifteen gauge needle connected to a short piece of plastic tubing. Continuous sampling was carried out from the beginning to sixty seconds after injection. The blood was collected in paraffined test tubes containing one drop of dried heparin solution. The injection was accompanied by a verbal signal to a second assistant who began to call off second intervals from a stopwatch. The end of the plastic tubing was then moved from tube to tube at 2-second intervals until the timed collection period was completed. Immediately following the collection of samples the tubes were corked and the blood mixed with the heparin by gentle inversion. The samples were centrifuged and the supernatant plasma removed and analyzed for T-1824, SCN, and D_2O concentrations. A sample of the original or injected mixture was analyzed similarly.

The Evans blue concentrations were read in terms of optical density directly in a Coleman junior spectropho-

tometer. Thiocyanate was determined by the standard ferric nitrate method (10). The D_2O concentrations were determined by a spectrophotometric method based on the emission spectra of atomic hydrogen and deuterium in a high frequency electrodeless discharge (11). This method is capable of measuring concentrations of deuterium as low as 1 part in 6500 with an accuracy of 5 per cent and with correspondingly greater accuracy at the high concentrations measured in these experiments. The pulmonary blood flow for each patient was calculated from the T-1824 dye curve according to the method of Hamilton, Moore, Kinsman, and Spurling (12). The pulmonary water flow was calculated from the plasma flow by correcting for the hematocrit and the known water content of human plasma and red cells (7). The concentrations of D_2O and SCN to be expected, had there been no transcapillary diffusion, were calculated from the formula reported elsewhere (7). The formula for SCN was modified for incomplete penetration of red cells as determined by a separate *in vitro* determination. The penetration was found to be 70 rather than 100 per cent, and hence the hematocrit was multiplied by 0.7 in calculating the expected concentrations of thiocyanate.

The difference between these expected concentrations and the measured concentrations was expressed as per cent of expected concentration and plotted against time for each patient. The extravascular D_2O space was calculated according to a method to be discussed later.

RESULTS

Deuterium oxide—normal subjects

In the normal subjects (Table I) the initial loss of D_2O in the samples averaged 42 per cent of the expected concentrations. These values were obtained at a point on the ascending limb of the dye curve where the concentrations were sufficient to insure accurate measurement. This loss decreased rapidly and the expected and observed concentrations soon approached each other. In fact, within

TABLE 1

Loss of deuterium oxide from the capillaries of the lung in normal subjects

Subject	Time after injection (*seconds*)	Hematocrit	T-1824 conc. in injecta (*opt. dens.*)	D_2O conc. in injecta (%)	T-1824 conc. (*opt. dens.*)	Expected D_2O concentration C_e (%)	Measured D_2O concentration C_o (%)	Per cent loss D_2O	Slope loss *vs.* time curve (°)	Appearance time of T-1824 (*sec.*)	100% loss time (by extrapolation)
R. P.*	16	32	14.35	70.40	.280	1.02	.56	45			
	22				.590	2.14	1.96	8			
	24				.546	1.98	2.00	− 1	70	12	8
	28	32			.338	1.23	1.40	−14			
F. H.	25	41	15.65	66.20	.380	1.07	.64	40			
	31				.724	2.04	1.88	8			
	35	42			.448	1.27	1.91	−51	78	18	20
	39	42			.184	.51	.99	−93			
C. G.	19	41	21.85	51.60	.676	1.08	.69	36			
	21				.736	1.17	.87	26			
	23				.608	.96	.95	1	77	10	10
	29	41			.112	.16	.30	−85			
W. W.	12	42	23.10	50.50	.244	.35	.16	54			
	14				.430	.62	.36	42			
	16				.470	.68	.52	24	81	9	9
	20	42			.222	.32	.48	−50			
G. Y.	17	45	26.25	58.50	.470	.66	.39	41			
	19				.700	.99	.65	35			
	21				.658	.94	.76	19	78	11	12
	25	45			.344	.49	.58	−20			
S. P.	18	44	26.00	58.64	.560	.81	.45	45			
	20				.742	1.08	.67	38			
	22				.480	.70	.73	− 5	83	13	16
	24	44			.264	.38	.52	−37			
M. E.	11	36	74.50	36.14	.640	.22	.14	38			
	13				1.270	.44	.36	18			
	15				.996	.35	.38	−10	83	6	8
	17	36			.546	.19	.28	−48			
	19				.296	.10	.16	−59			

* Anemia but no evidence of pulmonary or cardiac disease.

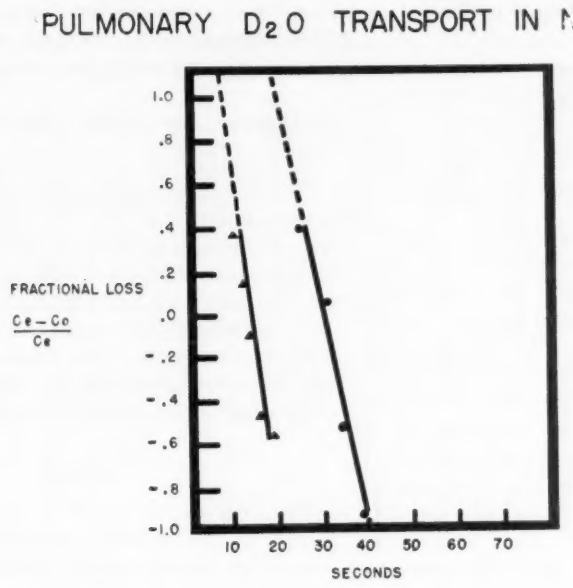

FIG. 1. CHART SHOWING FRACTIONAL LOSS D_2O *vs.* TIME FOR M. E. (DIAMONDS) AND F. H. (SQUARES) REPRESENTATIVE NORMAL SUBJECTS

Dotted line represents extrapolation. C_e represents expected concentrations based on T-1824 dye, C_o represents observed concentrations. Time is in seconds after dye appearance.

a few seconds after the peak dye concentration the collected samples contained more D_2O than the calculated expected concentrations. This implied that D_2O was returning to the circulation from some extravascular source. The curve representing the per cent loss of D_2O with respect to time seemed to be a straight line for each case and with approximately the same slope in all normal patients (Table I and Figure 1).

The curves of D_2O loss were extrapolated back to the appearance time of the dye T-1824. In all of the cases the extrapolation indicated a loss of 100 per cent D_2O at or near the appearance time (Table I and Figure 1).

Deuterium oxide—cardiac failure patients

In the patients with pulmonary congestion the slope of D_2O loss showed a similar straight line relationship in time (Figure 2). Most of these curves also extrapolated back to 100 per cent D_2O loss near appearance time. It can be seen, however, that the slope of these curves was less than in the normal subjects (Figure 2 and Table II). The average slope for the cardiac failure patients was 60 degrees as compared to 79 degrees for the normal subjects.

Calculation of pulmonary extravascular water space

Based on what seems to be a valid assumption that the D_2O exchange is complete in one circulation (see discussion) it is possible to calculate the size of the extravascular D_2O space for each patient: Let C_o = observed concentrations at time t of D_2O and C_e = expected concentration at time t of D_2O had there been no transcapillary diffusion, then

$$\int_{t_a}^{t_{equil.}} F\,(C_e - C_o)\,dt = \text{amount of } D_2O \text{ in extravascular space} = Q$$

where $t_{equil.}$ = time after appearance when $C_e = C_o$, and t_a = appearance time, and F = pulmonary water flow.

Since the concentration ($C_{equil.}$) of D_2O in the extravascular D_2O space at $t_{equil.}$ is

$$\frac{Q}{\text{Volume extravascular space}},$$

then by rearrangement:

$$\text{Volume extravascular space} = \frac{Q}{C_{equil.}}.$$

This volume can then be calculated from Q, obtained by integration of the curve of $C_e - C_o$ multiplied by the pulmonary water flow (calculated from the T-1824 curves), and $C_{equil.}$ which is the concentration when $C_e = C_o$. The volumes of the pulmonary extravascular space calculated by this method averaged 190 cc. (range 105 to 360 cc.) in the normal subjects and 290 cc. (range 113 to 330 cc.) in the patients with pulmonary congestion (Table III). When the volumes of the extravascular D_2O spaces in the normal subjects were plotted against the pulmonary blood flows (Figure 3), it appeared that the size of the space was a monotonic increasing function of the blood flow. This relationship did not hold, however, in the patients with pulmonary congestion.

Thiocyanate ion—normal subjects and cardiac failure patients

The thiocyanate pulmonary capillary diffusion pattern differed markedly from that of heavy water. In contrast to the large losses and constant diffusion pattern observed with the deuterium oxide, thiocyanate ion loss was negligible in the normals and only slightly higher in the patients with pulmonary congestion. No definite pattern of loss was discernible except for the relatively low values throughout the circulation period (Table IV).

DISCUSSION

The principle on which the method is based implies the use of a non-diffusible substance for comparison with the studied tracers. That Evans

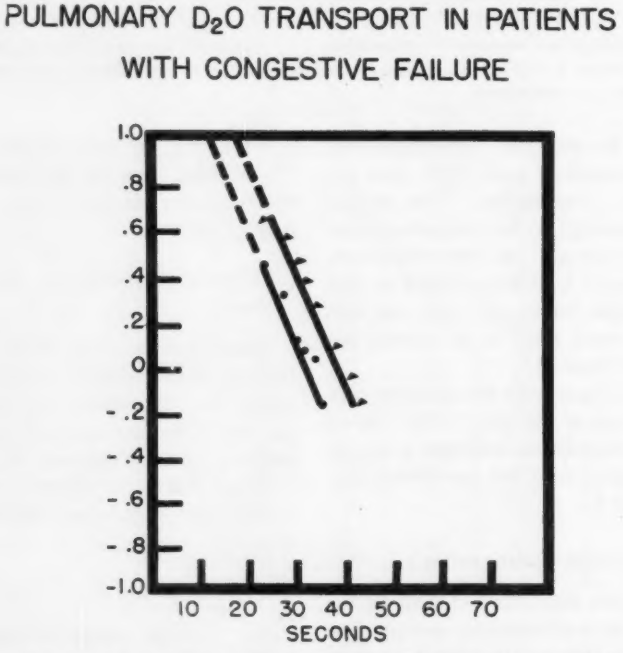

FIG. 2. CHART SHOWING FRACTIONAL LOSS D_2O *vs.* TIME FOR B. B. (CIRCLES) AND S. B. (TRIANGLES) REPRESENTATIVE PATIENTS WITH PULMONARY CONGESTION DUE TO HEART FAILURE

Dotted line represents extrapolation.

TABLE II

Loss of deuterium oxide from the capillaries of the lung in patients with pulmonary congestion due to heart failure

Patient and diagnosis	Time after injection (*sec.*)	Hematocrit	T-1824 conc. in injecta (*opt. dens.*)	D_2O conc. in injecta (%)	T-1824 concentration (*opt. dens.*)	Expected D_2O concentrations C_e (%)	Measured D_2O concentrations C_o (%)	Per cent loss D_2O	Slope of loss *vs.* time curve (°)	Appearance of time of T-1824 (*sec.*)	100% loss time (by extrapolation)
J. D.	30	37	38.10	46.70	.188	.16	.10	38			
Hypertensive heart	32				.258	.22	.16	30			
disease	36				.348	.30	.25	17	60	22	12
Congestive failure	38	37			.368	.32	.28	11			
	42				.318	.28	.32	−16			
R. F.	27		33.80	43.68	.512	.47	.32	32			
Acute myocardial	29	36			.598	.55	.43	22			
infarction	31				.558	.51	.44	15	67	14	14
Congestive failure	35				.402	.37	.40	− 7			
	39	36			.200	.19	.23	−22			
H. B.	41		32.00	47.09	.174	.16	.09	45			
Arteriosclerotic heart	45	45			.300	.28	.16	41			
disease	49				.384	.36	.22	38	46	33	24
Congestive failure	57				.460	.43	.34	21			
	61	45			.436	.41	.36	11			
	65				.400	.37	.38	− 2			
B. B.	24		33.80	53.70	.500	.59	.33	44			
Hypertensive heart	28	33			.780	.92	.64	31			
disease	30				.720	.85	.72	15	68	16	14
Congestive failure	32	33			.680	.80	.72	10			
	34				.528	.62	.60	4			
	36				.420	.49	.56	−13			
S. B.	24	25	32.40	49.75	.256	.31	.12	62			
Hypertensive heart	28				.580	.72	.32	56			
disease	30				.680	.85	.44	48			
Congestive failure	32				.680	.85	.52	38	68	18	19
	34	25			.616	.77	.56	27			
	36				.540	.67	.58	13			
	40				.428	.53	.54	− 2			
	42	25			.370	.46	.52	−13			
D. S.	55		32.40	46.52	.296	.30	.24	19			
Arteriosclerotic heart	57				.332	.33	.29	12			
disease	59	38			.388	.39	.33	15			
Congestive failure	61				.428	.43	.39	8	47	40	25
	63				.444	.44	.39	12			
	65				.444	.44	.44	1			
	67				.440	.44	.44	0			
	69				.408	.41	.40	2			
	71				.380	.38	.40	− 5			
	73	38			.352	.35	.38	− 8			
	75				.320	.32	.36	−13			
R. S.	23	49	34.50	45.89	.336	.27	.18	30			
Arteriosclerotic heart	25				.450	.36	.28	21			
disease	27				.444	.35	.30	15	67	18	10
Congestive failure	29	49			.390	.31	.30	3			
	37				.282	.24	.26	− 8			

blue dye is practically non-diffusible in one circulation through the normal lung seems to be a valid assumption (13, 14). Studies are now in progress to see if this is equally valid in heart failure, but the close agreement between cardiac outputs calculated from Evans blue dye curves and by the direct Fick method would seem to lend support to such an assumption.

The results obtained for deuterium oxide loss in the lung differed markedly from those previously reported for the forearm capillaries (7). In the forearm more than 95 per cent of the circulating D_2O is lost from the capillary bed throughout the first circulation period with minimal change in this loss during that time. By contrast, in the lungs initial losses were large but this was followed within a few seconds by significant gains of D_2O into the blood stream. This pattern observed in both normal subjects and congestive failure patients implied a relatively small extravascular tissue space in the lungs as compared with the forearm.

The estimation of the pulmonary extravascular tissue space in the patients studied provides credible values (average 190 cc. in normal subjects and 290 cc. in patients with congestive failure). These values seem reasonable in view of the known lung weights and the histologic consideration that the lung is composed for the most part of capillaries and air spaces, with very little extravascular tissue. The assumption on which the calculation of the lung extravascular space is based is

TABLE III

Estimated extravascular D_2O space in normal subjects and patients with pulmonary congestion due to heart failure

Normal subjects			Heart failure patients		
Subject	Pulmonary blood flow (*ml./sec.*)	Extravascular D_2O space (*cc.*)	Patient	Pulmonary blood flow (*ml./sec.*)	Extravascular D_2O space (*cc.*)
R. P.	80	105	J. D.	150	330
F. H.	90	110	R. F.	115	320
C. G.	80	180	H. B.	50	350
G. Y.	120	225	B. B.	70	255
S. P.	180	360	D. S.	50	113
W. W.	155	275	R. S.	143	310
M. E.	135	111			
	Average	190		Average	290

that there is complete exchange of circulating D_2O with extravascular water during one circulation. This assumption seems to be valid for when the curves of loss of D_2O in the lung are extrapolated backward they cross the 100 per cent loss axis at or very near the appearance time.

A comparison of thiocyanate losses from the forearm capillaries previously reported (7) with those now found in the lung capillaries reveals a marked difference in transcapillary diffusion patterns. The losses in the forearm, when recalculated correcting for the incomplete red blood cell penetration, average about 70 per cent, with a slight decrease in this loss during the circulation period. By contrast, the losses in the lung were less than 10 per cent in the normal subjects and

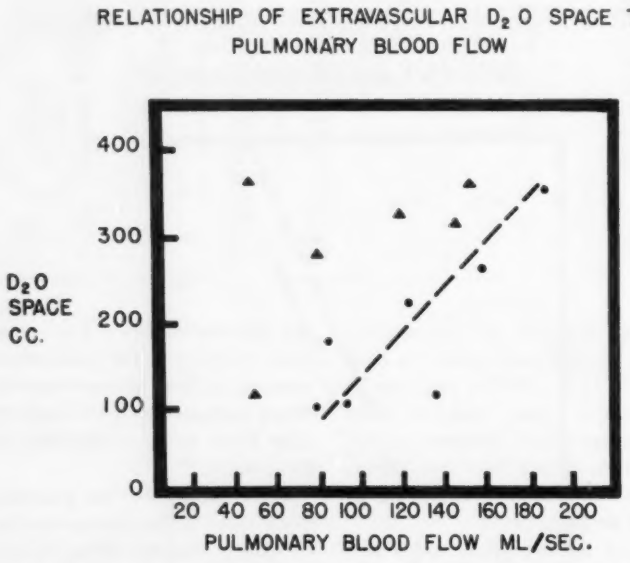

FIG. 3. CHART SHOWING PULMONARY EXTRAVASCULAR D_2O SPACE *vs.* PULMONARY BLOOD FLOW FOR NORMAL SUBJECTS (CIRCLES) AND PATIENTS WITH CONGESTIVE HEART FAILURE (TRIANGLES)

Dotted line represents apparent relationship in normal subjects. No relationship between size of space to blood flow is apparent in the patients with pulmonary congestion. Note also that all points representing extravascular D_2O space in congestive failure are above the line representing the normal subjects.

TABLE IV

Thiocyanate loss from the lung capillary bed in normal subjects and patients with pulmonary congestion due to heart failure

Normal subjects			Congestive failure patients		
Time after dye appearance (*seconds*)	Per cent loss of SCN (7 subjects)		Time after dye appearance (*seconds*)	Per cent loss of SCN (7 patients)	
	Average	S.D.		Average	S.D.
5	2.5	±7.4	5	8.5	±10.9
7	4.5	±4.1	7	14.5	±13.9
9	8.5	±5.9	9	11.4	± 3.7
11	9.3	±3.1	11	16.3	± 7.0
13	9.3	±7.4	13	11.7	± 5.3
			15	11.6	± 8.9
			17	9.7	± 4.6
			19	11.9	± 3.2
			21	12.3	± 4.8
			23	9.0	±11.6
			25	15.0	± 0.0
			27	10.7	± 4.5

less than 20 per cent in the patients with pulmonary congestion. Since the loss of thiocyanate is small *throughout* the circulation period it would seem to suggest an inherent difference in the permeability of the lung capillary walls to this ion as compared to the forearm. Should the small loss be due only to a very small interstitial space, it would be expected that the losses would be higher during the first part of the circulation rather than constantly small throughout. The slightly higher loss in the congested lung as compared with the normal lung can probably be explained on the basis of slight increase in permeability and/or slightly greater area available for diffusion. The relative impermeability of the lung capillaries to the electrolytes, Na^+ and I^- has been suggested by the work of other investigators (9), and seems, therefore, to be a characteristic of the pulmonary capillary bed.

The relation between the extravascular D_2O space and the pulmonary blood flow in the normal patients (Figure 3) suggests that the extravascular water space may be dependent upon the functioning capillary bed and may perhaps serve as an index of its size. Studies are now in progress in normal subjects to determine whether the extravascular D_2O space will change in the presence of induced variations in pulmonary blood flow.

SUMMARY

By employing a technique of injection into a basilic vein or pulmonary artery of a mixture of non-diffusible and diffusible substances and sampling during the period of first circulation from the femoral artery the following observations were made in man with regard to passage of deuterium oxide and thiocyanate ion through the pulmonary capillaries in seven normal subjects and seven patients with pulmonary congestion due to heart failure:

1. Deuterium oxide freely and rapidly passed through the pulmonary capillary walls.

2. When the transcapillary loss of D_2O was plotted against time, the curve obtained differed markedly from that found previously in the forearm (7) and indicated a small pulmonary extravascular tissue space.

3. Quantitative estimation of the extravascular water space of the lung indicated a mean value of 190 cc. in the normal subjects and of 290 cc. in the patients with congestive heart failure. In normal, but not in cardiac patients, the size of the pulmonary extravascular D_2O space appeared to be related to the pulmonary blood flow.

4. Thiocyanate ion in contrast to the previously observed large transfer through forearm capillaries left the pulmonary capillaries in only small amounts in the normal patients and in only slightly greater amounts in the patients with congestive failure. An analysis of these findings suggests a relative impermeability of the lung capillaries as compared to the forearm capillaries with respect to this ion.

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THE EFFECT OF GLASS UPON THE ACTIVITY OF THE VARIOUS PLASMA CLOTTING FACTORS

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Contact of the blood with a foreign surface such as glass initiates clotting not only by disruption of platelets but by the activation of plasma clotting factors. Many explanations have been advanced for the increased coagulability of the plasma. Contact with glass has been said to remove a lipid antithromboplastin (anti-cephalin) (1–3), to remove a protein inhibitor of prothrombin (4–6), to activate a plasma thromboplastin precursor (7–10), more specifically, the antihemophilic B factor (Christmas factor [3]) (11), and to convert an inactive precursor of prothrombin (prothrombinogen) to active prothrombin (12). Furthermore, the effect of glass has been attributed to minute amounts of thrombin adsorbed onto the glass (13). In a preliminary note (14), we said that contact of plasma with glass increases the activity of both the antihemophilic B factor and proconvertin. The present paper describes and discusses in greater detail the experiments which support this statement.

The idea of a plasma thromboplastin precursor activated by contact with a foreign surface is not new. Lenggenhager postulated in 1935 (15) that blood thromboplastic activity arose after contact of an inactive plasma precursor with a foreign surface in the presence of calcium. Lozner, Taylor, and MacDonald presented data in 1942 (16) which strongly suggested that glass could activate a plasma thromboplastin precursor in citrated plasma. Conley and Hartmann and their coworkers (7–9), and Quick and Epstein (10) presented further evidence for the glass activation of a plasma thromboplastin precursor. With the demonstration and characterization of two plasma thromboplastin precursors (17, 18) the question arose as to which one the glass influenced. The recent experiments of Biggs, Douglas, and Macfarlane (11) indicate it is the antihemophilic B factor.

There is also evidence that contact with glass activates a plasma factor not involved in blood thromboplastin formation. When a Quick "prothrombin time" is done upon plasma which has stood for several days in glass, and to which proaccelerin has been added to replace that which deteriorates during standing, the resultant clotting time may be shorter than that of the freshly prepared plasma. This cannot be due to the activation of a plasma thromboplastin precursor, for the excess brain extract thromboplastin which is added in a Quick test would mask this effect. Quick and Stefanini (12) and, more recently, Lewis and Ware (19) state that the shortening is caused by

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[3] There is no standard nomenclature for the newer clotting factors. The following terms are used in this paper. *Blood thromboplastin* is used in its classic meaning of an activity which arises during clotting and which is equivalent in effect to the clotting activity of aqueous tissue extracts. *Antihemophilic A factor* is the name used for the plasma thromboplastin precursor present in Seitz filtered or barium carbonate absorbed fresh normal plasma, absent from the plasma of most patients with hemophilia and not found in aged normal serum; synonyms are anti-hemophilic globulin and thromboplastinogen. *Antihemophilic B factor* is the name used for a different plasma thromboplastin precursor which is found in aged normal serum, is absent from the plasma of an occasional patient with otherwise classical hemophilia and is not found in Seitz filtered or barium carbonate adsorbed fresh normal plasma. Synonyms are plasma thromboplastin component (PTC), plasma thromboplastin factor-B (PTF–B), Christmas factor. *Proconvertin* is the name given to a stable prothrombin converting substance which can be demonstrated in normal plasma and serum. The terms used to describe proconvertin activity and the relation of proconvertin to convertin are discussed in detail in the body of this paper. Synonyms for proconvertin or for mixtures of proconvertin and convertin are SPCA, Factor VII, and Stable Factor. *Proaccelerin* is the name used for the labile accelerator of thrombin formation found in Seitz filtered or barium carbonate adsorbed fresh plasma but not found in aged serum; synonyms are plasma AC globulin and Labile Factor. *Accelerin* is the name used for the increased accelerator activity which is produced by adding a small amount of thrombin to adsorbed plasma; synonym is serum AC globulin.

the activation of prothrombin, a return to the early hypothesis of Bordet that prothrombin circulates in an inactive form which must be altered by contact with a foreign surface before clotting will occur. However, Alexander and Landwehr (20), who investigated the same phenomenon, explain the shortening by the evolution of proconvertin activity (SPCA) due not to an effect of the glass but to a slow "coagulation" of the plasma on standing.

Biggs and Macfarlane state in their book (21) that contact with glass during clotting accelerates the formation of proconvertin (Factor VII) from a precursor in the plasma. This statement stems from the observation, reported earlier by Alexander, DeVries, and Goldstein (22), that the proconvertin activity of serum from blood which has clotted in silicone coated tubes is less than that of serum from blood clotted in contact with glass. Alexander also showed that the addition of tissue thromboplastin to the blood increases the proconvertin activity of the resultant serum. Therefore, the increased proconvertin activity of serum from blood clotted in contact with glass could be an indirect effect secondary to the greater blood thromboplastin formation in glass, rather than a direct effect of the glass upon proconvertin. In fact, in their recent paper which describes the effect of glass upon antihemophilic B factor activity (11), Biggs, Douglas, and Macfarlane reverse their former opinion and state that glass contact does not increase proconvertin (Factor VII) activity.

In our experiments the clotting behaviour of freshly drawn, citrated, platelet poor plasma exposed only to silicone surfaces was compared with that of plasma prepared the same but shaken for 15 minutes with approximately ¼ its volume of fine quartz glass powder. One group of experiments measured only the effect of this procedure upon the plasma thromboplastin precursors in one-stage assays modeled after that of Langdell, Wagner, and Brinkhous (23). In a second group of experiments strong brain extract thromboplastin was added to the plasma. This eliminated any manifestations of the effect of glass upon the plasma thromboplastin precursors. The clotting behaviour of the two types of plasma were compared in the specific one-stage systems of Owren and Aas (24, 25) for prothrombin, proconvertin, and proaccelerin.

REAGENTS

A. *For the assay of antihemophilic A and antihemophilic B factor activity*

Hemophilia A plasma was obtained from a patient with the classical clinical findings of severe hemophilia and a coagulation defect which was corrected by oxalated barium sulfate adsorbed plasma but not by old serum. *Hemophilia B plasma* was obtained from a patient who also exhibited the classical clinical findings of severe hemophilia but whose coagulation defect was corrected by hemophilia A plasma and by old serum but not by oxalated barium sulfate adsorbed plasma. Blood from these patients was collected directly into chilled, silicone coated centrifuge tubes which contained 1 volume of 0.1 M sodium citrate for each 9 volumes of blood and centrifuged at 2,500 rpm and 2°C. for 40 minutes. The plasma was then transferred to lusteroid tubes with silicone coated pipettes and centrifuged for about 20 minutes at either 10,000 or 20,000 rpm in an ultracentrifuge. The resultant platelet poor plasma was mixed with ¼ its volume of fine quartz glass powder (Quarz, gewaschen u. gegluht, E. Merck, Darmstadt, which had been ground in a glass mill to give particles of diameter less than 0.068 mm.). After 15 minutes the quartz powder was removed by centrifugation at 2,500 rpm for 10 minutes. The "glass activated" hemophilic plasmas were then divided into small aliquots which were stored frozen at −20°C. for periods up to three months. The plasmas were used within about an hour after defrosting. They were checked and found to have a normal content of prothrombin and proconvertin and a slightly reduced but adequate amount of proaccelerin.

Normal plasma. Nine volumes of venous blood were collected directly into a chilled, silicone coated centrifuge tube which contained 1 volume of 0.1 M sodium citrate, and centrifuged for 40 minutes at 2,500 rpm at 2°C. A portion of the resultant platelet poor plasma was transferred to a silicone coated tube for use as the sample of *"silicone" plasma.* The remaining plasma was shaken with quartz glass powder as described above to prepare the sample of *"glass activated" plasma.* On one occasion the glass activation was accomplished by the addition of 10 small glass beads (diameter 4 mm.) to 2 ml. of plasma followed by continuous shaking for 15 minutes. All plasma samples were tested within about 4 hours after the blood was drawn.

Adsorbed ox plasma. Citrated ox plasma was filtered once through a 20 per cent asbestos filter and twice through a 50 per cent asbestos filter using a technique published earlier (24). Such plasma is free of prothrombin, proconvertin, and antihemophilic B factor but contains large amounts of proaccelerin, antihemophilic A factor, and fibrinogen.

"Cephalin." An acetone insoluble, ether extract of human brain was prepared according to the technique of Milstone (26). A description of our preparation has been given elsewhere (27). Small aliquots of a 2.2 gm. per cent suspension of lipid in veronal buffer were stored frozen at − 20°C. Before use these were diluted 1 to 20 or 1 to 30 with veronal buffer to give a 0.11 gm. per cent or a 0.07 gm. per cent lipid suspension.

Citrate-saline. This was made by mixing 1 part of 0.1 M sodium citrate with 5 parts of physiological saline.

Veronal buffer. A modified veronal buffer of pH 7.35 and ionic strength 0.154 was prepared as described elsewhere (27).

Calcium. A 0.030 M solution of calcium chloride was used.

B. *For the measurement of prothrombin-proconvertin (P & P), prothrombin, proconvertin, and proaccelerin activity*

Normal "silicone" and "glass activated" plasmas were prepared as above.

Proconvertin deficient plasma was obtained from a patient with a congenital deficiency of this factor. This plasma has a normal content of prothrombin and proaccelerin but only about 1 per cent proconvertin. The clotting behaviour of this plasma has been described in detail previously (25). Citrated plasma was stored in small lots at − 20°C. and defrosted as needed.

Adsorbed ox plasma for use as *proaccelerin* reagent was prepared as described above but was filtered only once through 50 per cent asbestos. *Accelerin* reagent was prepared by adding a small amount of thrombin to the ox plasma. Dried thrombin ("Hemoclaudan," Lövens Kemiske Fabrik, Copenhagen) was made up in about 5 ml. of veronal buffer. Several drops were added to about 10 ml. of ox plasma and the resultant fibrin clot removed after several minutes. This dropwise addition of thrombin was repeated until no further fibrin formed. The defibrinated ox plasma was than incubated at 37°C. for 30 minutes to inactivate the thrombin. This accelerin reagent gave a clotting time of 16 seconds in the proaccelerin system compared to a time of 25 seconds before the thrombin activation.

Serum. Prothrombin "free" serum was prepared by the addition of 0.3 ml. of saline brain extract thromboplastin for each 10 ml. of blood immediately after withdrawal followed by vigorous stirring for 10 minutes. After one hour the serum was removed and aged for 7 days at refrigerator temperature. It was then citrated with 1 volume of 0.1 M sodium citrate per 5 volumes of serum and stored at − 20°C.

Saline brain extract thromboplastin. A saline-buffer suspension of human brain was prepared by a method previously described (28).

Diluting fluid for the test plasmas was made by mixing 177 ml. of 0.034 M sodium citrate, 200 ml. of veronal buffer and 673 ml. of physiological saline.

Calcium. A 0.030 M solution of calcium chloride was used.

METHODS

A. *For the assay of the antihemophilic A and B factors*

When either normal or hemophilic plasma is recalcified in the presence of a strong saline brain extract thromboplastin, a clotting time of about 12 seconds is obtained. When a dilute ether extract of brain, the usual "cephalin" preparation, is substituted for the saline extract, normal plasma, after contact with glass, will clot in 40 to 80 seconds. The "cephalin" is an incomplete thromboplastin which is thought to supply only the equivalent of the platelet lipoid factor. The extra time is consumed by a preliminary reaction between the "cephalin" and the antihemophilic A and B factors to form plasma thromboplastin. When "cephalin" is added to hemophilic plasma which has been in contact with glass, a clotting time of 150 to 300 seconds is usually obtained. The longer clotting time is caused by a deficiency of one of the plasma thromboplastin precursors in the hemophilic plasma.

The long "cephalin" clotting time of hemophilic plasma can be shortened by adding dilute normal plasma to the hemophilic plasma. This is the basis for the assay for "anti-hemophilic factor" proposed by Langdell, Wagner, and Brinkhous (23). Their method may be used to assay either the antihemophilic A factor or the antihemophilic B factor by choosing either hemophilia A or hemophilia B plasma as the starting reagent. Furthermore, if these hemophilic plasmas are shaken with glass powder in their preparation they should each provide a relative excess in "glass activated" form of every plasma clotting factor except the plasma thromboplastin precursor which each lacks. The hemophilic plasmas can then be used as reagents to measure the effect of glass activation upon the antihemophilic factors. For example, hemophilia B plasma, after exposure to glass powder, should contain every plasma clotting factor in "glass activated" form except the antihemophilic B factor. Therefore, the determination of the shortening produced in the "cephalin" clotting time of the "glass activated" hemophilia B plasma by the addition of a given dilution first of normal "silicone" plasma and then of normal "glass activated" plasma should be a measure of whether or not exposure to glass changes the activity of the antihemophilic B factor.

Our procedure was to mix, in the order listed, 0.2 ml. of "cephalin," 0.2 ml. of "glass activated" hemophilia A or B plasma and 0.2 ml. of a dilution of either "silicone" or "glass activated" normal plasma. The mixture was incubated at 37°C. for 3 to 5 minutes and then 0.2 ml. of calcium chloride was added. In the antihemophilic A factor assay the normal plasma was diluted only in citrate-saline solution. In the antihemophilic B factor assay the final 1 to 5 dilution of normal plasma was made in adsorbed ox plasma to provide extra antihemophilic A factor and proaccelerin. When the effect of "silicone" plasma was being measured, only silicone technique was used.

The clotting times in both assay systems often varied with different normal plasmas. Therefore, a "normal"

dilution curve was not used to calculate different samples as per cent of normal. Each plasma was compared only with itself. For each plasma a 1/10 dilution of "silicone" plasma was compared with a 1/10 dilution of "glass activated" plasma and with at least one other dilution of the "glass activated" plasma. This gave rough quantitative meaning to differences in the clotting times of the 1/10 dilutions of the two kinds of plasma.

B. *For the measurement of the prothrombin-proconvertin (P & P) time, the proconvertin time, the prothrombin time and the proaccelerin time*

The principles and methods for the one stage determination of the P & P time, the proconvertin time, the prothrombin time, and the proaccelerin time have been described in detail previously (24, 25).

The *P & P time* is obtained by mixing 0.2 ml. of a 1/10 dilution of test plasma with 0.2 ml. of adsorbed ox plasma, 0.2 ml. of brain thromboplastin and 0.2 ml. of calcium chloride. Every known clotting factor is then in excess except for prothrombin and proconvertin whose content in the test plasma is measured.

The original *proconvertin time* method has been modified by the use of plasma from a patient with congenital hypoproconvertinemia. The assay system now consists of 0.2 ml. of hypoproconvertinemia plasma (supplies prothrombin in excess), 0.2 ml. of adsorbed ox plasma (supplies proaccelerin in excess), 0.2 ml. of a 1/10 dilution of the test plasma, 0.2 ml. of brain thromboplastin and 0.2 ml. of calcium chloride. The clotting time then reflects the proconvertin content of the test plasma.

The specific *prothrombin time* is determined by mixing 0.2 ml. of specially prepared prothrombin "free" serum (supplies proconvertin in excess), 0.2 ml. of ox plasma (supplies proaccelerin in excess), 0.2 ml. of a 1/10 dilution of the test plasma, 0.2 ml. of brain thromboplastin and 0.2 ml. of calcium chloride. Variation in the clotting time of this system will depend only upon the prothrombin content of the test plasma.

The *proaccelerin time* is measured by mixing 0.2 ml. of parahemophilia plasma (supplies prothrombin and proconvertin in excess), 0.2 ml. of a 1/20 dilution of the test plasma, 0.2 ml. of brain thromboplastin and 0.2 ml. of calcium chloride. The clotting time obtained will vary with the proaccelerin content of the test plasma.

RESULTS AND DISCUSSION

A. *The effect of contact with glass upon the activity of the antihemophilic A and B factors*

A representative sample of the data secured when normal "silicone" and "glass activated" plasmas were compared in the antihemophilic A factor assay are given in Table I. These data show no consistent difference between the clotting times obtained when a 1/10 dilution of either "silicone" or "glass activated" plasma is added to hemophilia A plasma. An occasional plasma, *e. g.*, samples 1 and 5, shows some suggestive increase in activity after "glass activation," but the majority of clotting times are the same for either type of plasma.

TABLE I

A comparison of the clotting times of "silicone" plasma and "glass activated" plasma in the hemophilia A assay system

	Clotting time (*seconds*)				
	Material added to hemophilia A plasma				
	"Silicone" plasma	"Glass activated" plasma			Citrate-saline control
Sample number	1/10	1/5	1/10	1/100	
1	123,122		107,104	142,140	162,154
2	109,110		104,103	136,142	
3	119,118		112,116	138,143	
4	104,100		105,103	136,133	
5	103,95	65,63	81,83	120,120	135,140
6	97,99	82,86	97,98	128,131	
7	110,109	91,89	106,107	136,140	
8	106,103	84,86	105,105	137,141	
9	90,89	64,64	80,86	117,118	141,143
10	95,93	89,90	99,96	125,123	
11	104,102	81,86	98,99	129,132	
12	100,97	94,88	102,97	132,121	
13	104,105	84,85	101,101	141,140	135,137
14	104,101	88,88	103,103	140,133	

Table II summarizes data obtained when normal "silicone" and "glass activated" plasmas were compared in the antihemophilic B factor assay. In contrast to the data of Table I, normal "glass activated" plasma was consistently more effective than normal "silicone" plasma in shortening the clotting time of hemophilia B plasma. Moreover, a comparison of the clotting times of the 1/100 dilution of "glass activated" plasma with the 1/10 dilution of "silicone" plasma shows that glass contact may increase antihemophilic B factor activity more than ten-fold. These findings confirm those of Biggs, Douglas, and Macfarlane (11) and suggest that the antihemophilic B factor may exist in the blood largely, and perhaps entirely, in an inactive form.

Recently, Tocantins (3) has stated that there is only one plasma thromboplastin precursor. This is covered by an inhibitor which contact with a foreign surface removes. Tocantins believes that in hemophilia A there is a normal amount of this thromboplastin precursor but an excess of inhibitor, whereas in hemophilia B the plasma is deficient in both the thromboplastin precursor and the inhibitor.

This theory will not fit our observations. "Glass activated" hemophilia A plasma still possesses an abnormally long "cephalin" clotting time which is corrected equally well by normal "silicone" and "glass activated" plasma. If there were only one plasma thromboplastin precursor, partly inactivated in its native state by an inhibitor absorbed by glass, then "glass activated" plasma should be more effective than "silicone" plasma in both the antihemophilic A and the antihemophilic B factor assay. Furthermore, asbestos filtered ox plasma contains no antihemophilic B factor but has a normal content of antihemophilic A factor which would have been activated by the asbestos (1). However, the addition of a 1/5 dilution of adsorbed ox plasma to normal "silicone" plasma will not shorten its long "cephalin" clotting time of about 200 seconds (see Table IV). In contrast, the addition of a 1/5 dilution of "glass activated" hemophilia A plasma produces an appreciable shortening. Our observations, therefore, add to the evidence that at least two plasma thromboplastin precursors exist, only one of which, the antihemophilic B factor, is activated by glass.

B. *The effect of glass upon proconvertin activity*

1. *The evidence that glass activates a factor in addition to the antihemophilic B factor*

The clotting activity of strong brain thromboplastin is equivalent to the combined effect of platelet lipoid factor, antihemophilic A factor and antihemophilic B factor. Therefore, a difference in the clotting times of "silicone" and "glass activated" plasma with excess strong brain thromboplastin, as in a Quick "prothrombin time," must mean that glass activates a factor in addition to the antihemophilic B factor. It also implies that the activation of this second factor does not depend upon the activation of the antihemophilic B factor nor upon the generation of a small amount of blood thromboplastin. The data in Table III support this view for they show that glass powder can shorten the Quick "prothrombin time" of normal platelet rich, normal platelet poor, and both types of hemophilic plasma. (In this and subsequent tables the clotting times are averages of at least two determinations and are in seconds.)

TABLE II

A comparison of the clotting times of "silicone" plasma and "glass activated" plasma in the hemophilia B assay system

Sample no.	Clotting time (*seconds*)			
	Material added to hemophilia B plasma			
	"Silicone" plasma	"Glass activated" plasma		Adsorbed ox plasma control
	1/10	1/10	1/100	
1	116,113	76,81	104,102	190,187
2	114,114	54,51	85,81	189,187
3	99,102	59,61	92,93	
4	118,126	110,111	151,152	
5	154,161	104,99	—	199,199
6	137,131	78,80	—	
7	124,123	65,67	122,119	195,198
8	152,145	103,101	150,144	
9	154,153	120,119	165,166	
10	140,141	128,121	169,163	
11	124,116	96,105	137,133	205,209
12	166,162	127,134	166,166	

TABLE III

The Quick "prothrombin time" of "silicone" and "glass activated" plasmas deficient in different precursors of blood thromboplastin

Type of plasma	Clotting time (*seconds*)	
	"Silicone"	"Glass activated"
Normal plasma		
Platelet rich	17.4	11.2
Platelet poor*	—	11.4
Hemophilia A	12.2	9.6
Hemophilia B		
Platelet poor*	18.4	12.8

* Centrifuged at 10,000 rpm for 40 minutes.

The clotting times of "silicone" and "glass activated" samples of normal plasma recalcified in the presence of three different "thromboplastic" agents are given in Table IV. The large difference in the "cephalin" clotting times reflects the fact that "cephalin" reacts with the antihemophilic B factor. The variation which glass can cause in the "cephalin" clotting time of normal plasma must be remembered when this test is used, as advocated (23), for the presumptive diagnosis of hemophilia. However, the main point of this table is to show that the longer clotting time of "silicone" plasma which persists in the presence of strong brain thromboplastin disappears, as Tocantins noted (1, 2), when Russell viper venom is the "thromboplastic" agent. We have shown (29) that the clotting activity of Russell viper venom is independent of the proconvertin content of plasma. Therefore, if the shortening produced by glass is not manifest in the presence of viper

TABLE IV

The clotting times of "silicone" and "glass activated" plasma when "cephalin," brain thromboplastin and Russell viper venom are used as the "thromboplastic" agents

Thromboplastic agent	Clotting time (*seconds*) "Silicone" plasma	"Glass activated" plasma
"Cephalin" 0.11 gm. %	197	36.5
Saline brain extract	14.5	9.5
Russell viper venom	9.5	10.5

venom, suspicion arises that the shortening is due to proconvertin activation.

2. *The proof that glass increases proconvertin activity*

Examples are given in Table V of the results obtained when normal "silicone" and "glass activated" plasmas were analyzed in the P & P, proconvertin, prothrombin, and proaccelerin systems. These data show that the shortening produced by glass in the presence of strong brain thromboplastin is due to the activation of proconvertin. When, as in the prothrombin and proaccelerin systems, an excess of proconvertin in other reagents masks a change in the proconvertin content of the test sample, no difference is found between "silicone" and "glass activated" plasma.

As Table VI shows, a comparable increase in proconvertin activity was obtained when hemophilia A, hemophilia B, and parahemophilia plasmas were tested. The activity of serum prepared by adding brain thromboplastin to blood and aged in glass for 5 days at 6°C. could not be increased by glass powder. However, glass could activate serum prepared in the same manner but stored frozen at − 20°C. for one year. When "silicone" congenital hypoproconvertinemia plasma was shaken with glass powder, its very long proconvertin time was reduced by the activation of the small amount of proconvertin which this plasma contains.

TABLE V

The effect of shaking with quartz glass powder upon the P & P, proconvertin, prothrombin and proaccelerin clotting times of normal plasma

Determination	Sample 1 "Silicone"	Sample 1 "Glass act."	Sample 2 "Silicone"	Sample 2 "Glass act."
P & P	31.0	26.0	30.5	24.0
Proconvertin	32.0	24.5	36.5	28.5
Prothrombin	30.0	32.0	31.5	32.0
Proaccelerin	36.5	35.0	29.0	29.5

The proconvertin times of varying dilutions of "silicone" and "glass activated" plasma were also measured when the dilutions were made not in diluting fluid but in congenital hypoproconvertinemia plasma. This provided an increasing relative excess of all clotting factors except proconvertin with increasing dilution of the test plasmas. The results, when plotted upon double logarithmic paper as in Figure 1, gave lines with parallel slopes. The relative difference between the "silicone" and "glass activated" plasmas persisted for all dilutions. Some workers have claimed that glass activates prothrombin (12). At the 1 per cent dilution the contribution of the prothrombin in the test plasma to the total prothrombin in the clotting mixture is insignificant yet the relative difference between the clotting times of "silicone" and "glass activated" plasma is as great as with the undiluted samples which provide 50 per cent of the prothrombin in the clotting mixture. In our opinion, this experiment eliminates the possibility that the factor activated by glass and measured in the presence of strong brain extract can be anything but proconvertin.

TABLE VI

The effect of shaking with quartz glass powder upon the proconvertin time of different kinds of plasma and serum

Material	Proconvertin clotting time (*seconds*) "Silicone" plasma	"Glass activated" plasma
Hemophilia A plasma	34.5	24.0
Hemophilia B plasma	36.5	26.5
Parahemophilia plasma*	31.5	26.5
Serum aged 5 days at 6°C.	29.5	29.5
Serum frozen −20°C. for 1 year	35.5	30.0
Congenital hypoproconvertinemia plasma	107	79.5

* "Silicone" parahemophilia plasma was not available. The comparison had to be made between parahemophilia plasma taken and stored frozen in glass and analyzed immediately after thawing with the same plasma shaken with glass powder before analysis.

3. *The extent of the increase in proconvertin activity produced by glass powder*

The extent of the increase in proconvertin activity which glass powder may produce is meas-

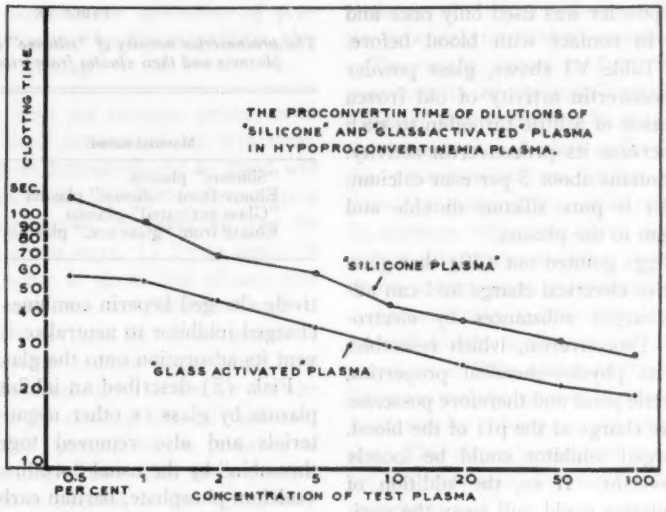

FIG. 1.

ured best when the proconvertin content of the plasma has been depressed by Dicumarol®. In one group of experiments the effect of exposure to glass powder upon the Quick "prothrombin time" of "silicone" Dicumarol® plasma was compared with effect of adding prothrombin free serum to the Dicumarol® plasma. Quick "prothrombin times" were done upon the following combinations: 0.2 ml. of "silicone" plasma plus 0.2 ml. of buffer; 0.2 ml. of "glass activated" plasma plus 0.2 ml. of buffer; and 0.2 ml. of "silicone" plasma plus 0.2 ml. of prothrombin free serum. The striking shortening of the Quick time by glass powder is readily apparent from the data shown in the upper columns of Table VII. Glass activation approached the effect of adding serum to "silicone" Dicumarol® plasma.

The data in the lower columns of Table VII are the proconvertin times of the same "silicone" and "glass activated" Dicumarol® plasmas. The percentages were obtained from a dilution curve made with normal "silicone" plasma. These data show that glass activation can increase proconvertin activity about three-fold or greater and can mask a moderate reduction in the proconvertin content by Dicumarol®. All but one of these plasmas appear to contain an excess of proconvertin when compared with normal "silicone" plasma.

4. *The means by which glass powder activates proconvertin*

There is insufficient evidence to be certain of how glass increases proconvertin activity in citrated plasma. As discussed earlier, neither the prior activation of the antihemophilic B factor nor the generation of a small amount of blood thromboplastin is the mechanism. Minute amounts of thrombin, either as a contaminant on the glass or somehow formed in the citrated plasma on the addition of glass powder, can not be implicated.

TABLE VII

The extent of the increase in proconvertin activity of Dicumarol® plasma produced by glass activation

Type of test	Plasma sample				
Modified Quick test Clotting times (*sec.*)	1	2	3	4	5
"Silicone" plasma plus buffer	36.5	33.0	59.0	34.5	34.0
"Glass act." plasma plus buffer	23.0	23.5	36.5	26.0	22.0
"Silicone" plasma + serum	21.0	20.0	25.5	22.5	19.0
Proconvertin test					
"Silicone" plasma Clotting time (*sec.*)	42.0	47.0	59.5	43.0	38.5
Per cent	54	36	15	49	72
"Glass act." plasma Clotting time (*sec.*)	32.5	32.0	39.5	29.5	26.5
Per cent	130	150	66	190	300

The pure quartz powder was used only once and had never been in contact with blood before. Furthermore, as Table VI shows, glass powder increases the proconvertin activity of old frozen serum. The addition of a little thrombin to such serum did not increase its proconvertin activity. Ordinary glass contains about 5 per cent calcium, but quartz powder is pure silicone dioxide and can not add calcium to the plasma.

Gortner and Biggs pointed out (30) that glass possesses a negative electrical charge and can adsorb positively charged substances by electrostatic attraction. Proconvertin, which resembles prothrombin in its physico-chemical properties, has an acid isoelectric point and therefore possesses an overall negative charge at the pH of the blood. A positively charged inhibitor could be loosely bound to proconvertin. If so, the addition of glass powder to plasma could pull away the positively charged inhibitor from the main body of the proconvertin molecule.

This hypothesis gains theoretical support from the observation that heparin will prevent the activation of proconvertin by glass. Ware and Stragnell (31) had noted that the shortening of the "prothrombin time" of plasma which stands in glass tubes could be prevented by adding 100 micrograms of heparin to each ml. of the usual 0.1 M oxalate anticoagulant. The experiments summarized in Table VIII reveal that 50 micrograms of heparin per ml. of plasma will prevent or greatly reduce the activation of proconvertin by glass powder. The addition of heparin after the plasma has been shaken with glass powder does not "deactivate" previously formed active proconvertin. These findings could mean that the negatively charged heparin combines with a positively charged inhibitor to neutralize it and thereby prevent its adsorption onto the glass.

TABLE VIII

The effect of heparin (50 micrograms per ml. plasma) upon the proconvertin activity of "silicone" and "glass activated" plasma

Type of plasma	Proconvertin clotting time (*sec.*) Sample 1	2	3
"Silicone" plasma			
No heparin	34.0	31.0	34.0
Added heparin	36.0	31.5	36.0
"Glass activated" plasma			
No heparin	27.0	25.5	27.0
Heparin before glass act.	34.5	31.0	32.0
Heparin after glass act.	26.5	28.0	28.5

TABLE IX

The proconvertin activity of "silicone" and "glass activated" plasmas and their eluates from tricalcium phosphate

Material tested	Proconvertin clotting time (*sec.*)
"Silicone" plasma	37.0
Eluate from "silicone" plasma	35.0
"Glass activated" plasma	25.0
Eluate from "glass act." plasma	25.0

Fiala (5) described an inhibitor removed from plasma by glass or other negatively charged materials and also removed together with "prothrombin" by the usual "prothrombin" adsorbents (calcium phosphate, barium carbonate, *etc.*). Although Fiala was unable to elute the inhibitor from glass he was able to elute it from barium carbonate and to demonstrate its effects after acid destruction of the accompanying "prothrombin." Fiala and Roth originally believed that this inhibitor acted against prothrombin, but later stated (6) that it might act upon proconvertin (stable conversion factor).

Evidence that a proconvertin inhibitor is removed with proconvertin by the "prothrombin" adsorbent tricalcium phosphate is provided by the following experiment. "Silicone" and "glass activated" plasmas were each agitated with 2.6 mg. per ml. of tricalcium phosphate for ten minutes. Each tricalcium phosphate sediment, after washing, was mixed with a small amount of 0.2 M sodium citrate. Each citrate eluate was then made up with veronal buffer to the citrate concentration and volume of the original plasma. Silicone technique was used for the eluate from the silicone plasma. The proconvertin times of the original plasmas and their eluates were then compared. As shown in Table IX, the difference between the proconvertin activity of "silicone" and "glass activated" plasma was found to persist in their eluates. This finding, which strongly suggests that tricalcium phosphate adsorbes a proconvertin inhibitor with proconvertin from "silicone" plasma, leads us to believe that the activation of proconvertin by glass may be due to the adsorption onto the glass of the inhibitor described by Fiala.

5. *The relation between the activation of proconvertin by glass and by thromboplastin plus calcium during clotting*

Although glass does not increase proconvertin activity through blood thromboplastin formation, the addition of tissue thromboplastin to blood will increase the proconvertin activity of the resultant serum (22). Apparently proconvertin can be activated in at least two ways: 1) By an action of glass which can occur in decalcified plasma and therefore begins as soon as citrated blood is placed in a glass tube; and 2) by an action of thromboplastin which cannot begin until the plasma has been recalcified. The reason for the difference between the clotting time of "silicone" and "glass activated" plasma in the presence of strong brain thromboplastin is that some preactivated proconvertin is present in the "glass activated" plasma whereas the activation in "silicone" plasma by thromboplastin and calcium is not initiated until after recalcification.

The following experiment suggests that the same inhibitor is removed by glass on standing as by thrombolpastin plus calcium during clotting. Nine and one-half ml. of blood were collected into each of two silicone tubes. One tube contained 1/2 ml. of brain extract thromboplastin; the other contained 1/2 ml. of buffer. The contents of each tube were mixed by tilting once and stood undisturbed at 37°C. for one hour after clotting. The serums were transferred to silicone coated tubes which contained 0.2 ml. of sodium citrate per ml. of serum. The proconvertin activity of each serum was measured with silicone technique in a system modified by the substitution of accelerin reagent for the usual proaccelerin reagent. This eliminated differences due to the accelerin content of the two serums. Each serum was then shaken with 1/4 its volume of glass powder and the proconvertin content measured again.

TABLE X

The effect of shaking with quartz glass powder upon the proconvertin activity of serum prepared from blood clotted in silicone coated tubes with and without exogenous thromboplastin

Serum from blood clotted	Proconvertin clotting time (*sec.*)	
	Before "glass act."	After "glass act."
With added thrombopl.	17.0	17.0
Without thrombopl.	32.0	23.0

The results, shown in Table X, reveal that the proconvertin of "silicone" plasma is completely activated during clotting in the presence of strong thromboplastin. A glass surface is not needed for this activation and no further activation is produced by shaking the serum with glass powder. In contrast, when the blood clotted in a silicone coated tube without added thromboplastin, little or no activation of proconvertin occurred. This is because only a little blood thromboplastin is formed during clotting in "silicone" coated tubes for there is little active antihemophilic B factor. The proconvertin activity of this serum was greatly increased by subsequent shaking with glass powder. This strongly suggests that glass and thromboplastin remove the same inhibitor from proconvertin.

The increase in proconvertin activity produced during clotting by strong thromboplastin is greater than that which glass powder can produce. "Glass activated" plasma repeatedly gave a clotting time of about 22 seconds in this system, whereas the serum from blood clotted with exogenous thromboplastin gave clotting times in the 15 to 17 second range even after standing for six hours. It would appear as though glass adsorption is not capable of producing the maximum activation of proconvertin.

6. *Proconvertin nomenclature*

These experiments have led us to modify our terms for proconvertin activity. Proconvertin in blood before contact with glass appears to be combined with an inhibitor and may therefore be called *inactive proconvertin*. When blood is placed in a glass tube the inhibitor is slowly adsorbed onto the glass with the formation of increasing amounts of *active proconvertin*. Therefore, unless prepared with careful silicone technique, plasma will contain a mixture of *inactive proconvertin* and *active proconvertin*. Plasma examined soon after the blood is drawn, however, will contain mainly *inactive proconvertin*.

When blood is clotted with large amounts of thromboplastin two changes occur. The thromboplastin will convert the remaining *inactive proconvertin* to *active proconvertin*. In addition, some of the *active proconvertin* is believed to re-

act with the thromboplastin to form a new and more powerful activity which has been called *convertin*. *Convertin* is a qualitatively different activity as can be shown (27) by its immunity to the action of the lipid antithromboplastin of Tocantins (1–3). It is a *combination* of thromboplastin with *active proconvertin* in the presence of calcium and should not be confused with the clotting activity of a *mixture* of thromboplastin and *active proconvertin*.

Fresh serum from blood clotted with strong thromboplastin will contain a mixture of *convertin* and *active proconvertin*. The *convertin* activity is thought to disappear within a few hours, probably due to the presence of an anti-convertin. The serum will then contain only *active proconvertin*. In contrast, serum prepared by clotting blood in a silicone coated tube, that is, with no exposure to glass and with little thromboplastin formation, will contain primarily *inactive proconvertin*. This *inactive proconvertin* can be activated by glass contact.

This concept of *inactive* and *active proconvertin* seems a more useful way to think about proconvertin than to attempt a rigorous distinction between a plasma and serum form. For by variation of the conditions of preparation, either *inactive* or *active proconvertin* may predominate in either the plasma or the serum.

The fact that glass activates both the antihemophilic B factor and proconvertin does not have to mean that both substances circulate in combination with the same inhibitor. However, one is impressed with the similar properties of these two clotting factors. They are both adsorbed by the "prothrombin" adsorbents and eluted by sodium citrate. They both persist in serum where their activity is usually greater than in plasma. It may be that the same substance inhibits them both.

SUMMARY

1. The clotting behaviour of platelet poor plasma exposed only to silicone surfaces was compared with that of plasma which had been shaken with quartz glass powder. The effect of such glass activation upon the various plasma clotting factors was measured in one-stage assays for antihemophilic A factor, antihemophilic B factor, prothrombin, proconvertin and proaccelerin.

2. Exposure to glass increased the activity of the antihemophilic B factor and proconvertin. No change in antihemophilic A factor, prothrombin or proaccelerin activity was found.

3. Glass powder increased proconvertin activity three-fold or more. Moderate reductions in the proconvertin content of plasma due to Dicumarol® were masked.

4. Glass powder could not activate proconvertin in the presence of heparin, but heparin could not "deactivate" previously formed active proconvertin.

5. A possible mechanism for the glass activation of the antihemophilic B factor and proconvertin is the adsorption of a positively charged inhibitor onto the negatively charged glass.

6. The relation between the activation of proconvertin by glass and by thromboplastin plus calcium has been examined. Proconvertin has been defined as *inactive proconvertin* or *active proconvertin*, rather than as a plasma or serum activity.

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THE MECHANISM OF AMMONIA EXCRETION DURING AMMONIUM CHLORIDE ACIDOSIS [1]

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The response of urinary ammonia to strong acid loads has been repeatedly observed to follow a characteristic pattern (1–3). Initially, ammonia excretion increases to a slight extent, then slowly attains maximum intensity after three to four days, and is maintained at a rate which parallels the magnitude of the acid load. This enhanced rate of ammonia excretion has usually been ascribed to the accelerated transport of ammonia into the tubular urine as a result of a sharp fall in urine pH (4–8). Pitts, however, found that chronic acidosis resulted in the excretion of more ammonia at any given urine pH than did acute acidosis (5) and suggested that prolonged acidosis might stimulate certain adaptive changes in the ammonia-producing system. The subsequent demonstration by Davies and Yudkin (9) of increased activity of renal ammonia-producing enzymes in rats given acid loads strongly supports the thesis that the production of ammonia may also constitute a regulatory mechanism of ammonia excretion. Apparently, therefore, two processes control the excretion of ammonia: 1) The *production* of ammonia in the tubular cell by the action of various ammonia-producing enzymes on precursor amino acids; and 2) the *transport* of the ammonia thus produced from tubular cell into tubular urine.

The purpose of the present investigation was three-fold: 1) To determine the relationship between ammonia excretion and renal glutaminase activity during two circumstances: the early period of adaptation following the administration of an acid load, and the period of maximum ammonia excretion following the administration of different amounts of strong acid; 2) to define, if possible, the factors responsible for the adaptation of renal glutaminase; and 3) to determine the effect of urine pH upon the relationship between ammonia excretion and renal glutaminase activity.

PROCEDURE

Male Sprague-Dawley rats, weighing 350 to 400 gm., were tube fed 10 ml. of standard electrolyte deficient diet twice daily. Each 10 ml. of diet contained 1.0 gm. of protein, 0.75 gm. of fat, 1.0 gm. of glucose, essential salts and soluble vitamins, but no sodium, potassium, or chloride. Preliminary unpublished observations in this laboratory have shown that variations in dietary intake result in altered excretion of acid by the kidneys. In order to obviate variations in acid excretion arising from differences in dietary intake, rats were tube fed constant amounts of diet. All rats were kept in an air-conditioned room maintained at a constant temperature to avoid any change in the excretion of acid resulting from metabolic response to temperature changes.

The first group of experiments was undertaken to determine the relationship of glutaminase adaptation to the rise in ammonia excretion during the early phase of a constant acid load. In order to avoid variations in NH_3 excretion resulting from different antecedent diets, 32 rats were tube fed the standard electrolyte deficient diet for five days. At the end of this period 4 of these rats were sacrificed and their kidneys analyzed for glutaminase to serve as controls. The remainder then received 2½ mM of NH_4Cl twice daily; the rats were sacrificed in groups of 4 at 24, 36, 48, 72, and 96 hours, 6 days, and 14 days, and their kidneys analyzed for glutaminase activity. Daily twenty-four hour urine samples were collected and analyzed for pH, titratable acidity, and ammonia throughout the experiment.

To determine the relationship between ammonia excretion and renal glutaminase activity in rats receiving different acid loads, a second group of experiments was initiated. Seven groups of 4 rats were tube fed the standard electrolyte deficient diet (SEDD) containing varying amounts of acid. One group received only the SEDD. A second group received SEDD plus 6 mM $NaHCO_3$ daily. Four groups received SEDD plus 2, 3, 5, and 6 mM NH_4Cl daily, respectively. A final group received SEDD plus 6 mM NH_4Cl and 6 mM KCl daily. After 14 days (ammonia excretion having been constant for about eight days), the rats were sacrificed and the activity of renal glutaminase was determined.

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METHODS

Daily twenty-four hour urine samples from each rat were collected under mineral oil using phenyl mercuric nitrate and toluene as preservatives. The ammonia in the urine was determined by a modification of the microdiffusion method of Conway (10). The pH of the urine was measured with a Cambridge pH meter using external glass electrodes, and corrected to 37.5°C. by a factor of 0.01 pH unit per degree. The urine was titrated to corrected pH 7.4 with 0.1 N NaOH to determine the titratable acid.

At the end of each experimental period, the rats were anesthetized by an intraperitoneal injection of hexabarbital (Evipal® sodium) and arterial blood was collected anaerobically from the abdominal aorta. Muscle samples were removed from the hind legs. The kidneys were then removed and placed in chilled isotonic saline.

Maximum glutaminase activity was measured by adding 0.2 ml. of cold 5 per cent kidney homogenate (11) to a test tube containing 0.3 ml. of 0.25 M tris (hydroxymethyl) amino methane and 0.4 ml. of 0.6 M sodium phosphate (both solutions were adjusted to pH 8.0), 0.15 ml. of 0.12 M sodium cyanide, 0.25 ml. of 0.2 M glutamine and 0.2 ml. of water. The tubes were then incubated for 30 minutes at 38°C. The reaction was stopped by the addition of 0.15 ml. of 50 per cent perchloric acid. With each determination a blank was run in which the enzyme was replaced with water. The amount of ammonia produced was measured by nesslerization of an aliquot (12). Dry weights were determined by adding an aliquot of the 5 per cent homogenate to a weighed tube and drying for two or more days at 90°C. to a constant weight. The activity of glutaminase was expressed as micro moles of ammonia per 100 mgm. of dry kidney per hour.[3]

The muscle samples were weighed and dried; fat was removed with ether; electrolytes were extracted with 0.75 N nitric acid (13). The content of sodium and potassium in the serum and in the muscle extracts was determined on the flame photometer (14). Chloride was measured in the serum and extracts of muscle by the Hald modification of the ope.. ..arius method (15). The CO_2 content was determined on 0.2 ml. serum (15).

RESULTS

Following the start of the constant daily administration of 5 mM NH_4Cl (2½ mM every 12 hours) renal glutaminase activity remained unchanged for 24 hours, then rose in a stepwise manner, reaching maximum activity in approximately six days (Figure 1). The rise in ammonia excretion preceded the rise in glutaminase activity,

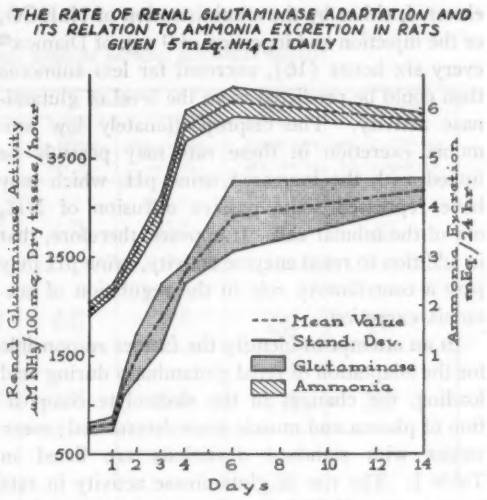

FIG. 1

showing a slight increase during the first 24 hours, but thereafter roughly paralleled the rise in renal glutaminase activity. The progressive rise in ammonia excretion during the early period of acid loading was, therefore, related to a similar rise in the activity of renal glutaminase.

In order to determine whether different loads of acid stimulated ammonia excretion by activating glutaminase, the ammonia excretion and glutaminase activity were compared in seven groups of rats, each group being maintained on a different acid load. Renal glutaminase activity was determined at the end of 14 days, when constant maximum ammonia excretion had been attained. Urine pH did not vary significantly among the rats receiving different loads of NH_4Cl. Under such circumstances, there was an excellent correlation between maximum ammonia excretion and the activity of renal glutaminase (Figure 2). During chronic acid loading the excretion of ammonia, therefore, appears to be more closely related to glutaminase activity than to urine pH.

The effect of increased urine pH upon the relationship between ammonia excretion and renal glutaminase activity is illustrated in Figure 3. The regression line was calculated from the data obtained in studies on rats receiving varying NH_4Cl loads; the urine pH did not vary significantly among these groups. Rats whose urine pH was

[3] It has been demonstrated (Copenhaver, unpublished results) that the conditions outlined here are optimal for the determination of phosphate-activated glutaminase in water homogenates of rat kidney.

elevated, either by the administration of $NaHCO_3$ or the injection and ingestion of 9 mg. of Diamox® every six hours (16), excreted far less ammonia than could be predicted from the level of glutaminase activity. The disproportionately low ammonia excretion in these rats may possibly be linked with the increased urine pH, which may have repressed some passive diffusion of NH_3 out of the tubular cell. It appears, therefore, that in addition to renal enzyme activity, urine pH may play a contributory role in the regulation of ammonia excretion.

In an attempt to identify the factors responsible for the adaptation of renal glutaminase during acid loading, the changes in the electrolyte composition of plasma and muscle were determined; mean values with standard deviations are listed in Table I. The rise of glutaminase activity in rats receiving 6 mM NH_4Cl daily was accompanied by a decrease in muscle potassium from 51.4 mEq. per 100 gm. fat-free dry tissue (FFDT) to 47.3 mEq. per 100 gm. FFDT. The addition of 6 mM of KCl to the diet (Group III) had no significant effect upon the response of glutaminase to the acid load. However, the muscle potassium of rats given KCl + NH_4Cl (Group III) was not significantly different from the muscle potassium of rats given NH_4Cl alone (Group II) owing to the large standard deviation of the former which resulted from a marked divergence from the mean

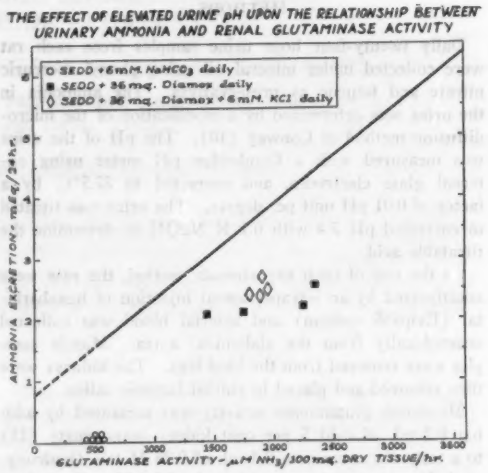
THE EFFECT OF ELEVATED URINE pH UPON THE RELATIONSHIP BETWEEN URINARY AMMONIA AND RENAL GLUTAMINASE ACTIVITY
○ SEDD + 6 mM. NaHCO3 daily
■ SEDD + 36 mg. Diamox daily
◇ SEDD + 36 mg. Diamox + 6 mM. KCl daily
AMMONIA EXCRETION - mEq./24 hr.
GLUTAMINASE ACTIVITY - μM NH3/100 mg. DRY TISSUE/hr.

FIG. 3

in one rat. In other studies on rats given Diamox® reported elsewhere (16) correction of the potassium deficiency was accomplished by the administration of KCl without depressing glutaminase activity. It appears likely, therefore, that changes in tissue potassium, as reflected by the potassium content of muscle, are not responsible for the adaptation of renal glutaminase. Although the rise of glutaminase activity in rats receiving 6 mM NH_4Cl was accompanied by a decrease in the serum bicarbonate content from 23.9 mEq. per L. in controls to 17.8 mEq. per L., no significant change in bicarbonate content supervened in rats given 2 mM NH_4Cl daily (Group IV) despite a significant rise in glutaminase activity. Therefore, changes in serum bicarbonate content can not be causally related to the adaptation of renal glutaminase.

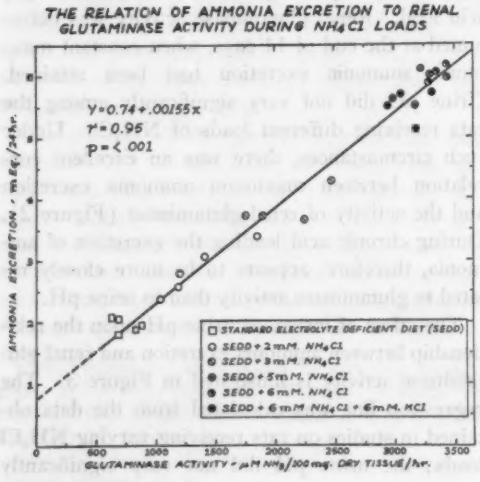
THE RELATION OF AMMONIA EXCRETION TO RENAL GLUTAMINASE ACTIVITY DURING NH4Cl LOADS
Y=0.74+.00155x
r=0.95
P=< 001
AMMONIA EXCRETION - mEq./24 hr.
GLUTAMINASE ACTIVITY - μM NH3/100 mg. DRY TISSUE/hr.
□ STANDARD ELECTROLYTE DEFICIENT DIET (SEDD)
○ SEDD + 2 mM. NH4Cl
◎ SEDD + 3 mM. NH4Cl
● SEDD + 5 mM. NH4Cl
● SEDD + 6 mM. NH4Cl
● SEDD + 6 mM NH4Cl + 6 mM. KCl

FIG. 2

DISCUSSION

The excellent correlation between ammonia excretion and the activity of renal glutaminase during both the early period of adaptation in rats given constant acid loads and the period after steady ammonia excretion had been attained in rats given different acid loads strongly suggests that the rate at which ammonia is excreted is, at least partially, regulated by the activity of the ammonia-producing enzymes. The activation of the NH_3-producing enzymes by the administra-

TABLE I

THE RELATION OF SERUM AND MUSCLE ELECTROLYTES TO AMMONIA EXCRETION AND RENAL GLUTAMINASE ACTIVITY

Group	Treatment	Duration Days	SERUM				MUSCLE		MEAN NH_3	GLUTAMINASE
			Na mEq./L	K mEq./L	Cl mEq./L	CO_2 mEq./L	Na_c mEq./100gm. fat-free dry wt.	K_c mEq./100gm. fat-free dry wt.	Excretions mEq./24 hr.	μM. NH_3/100 mg. dry tissue/hr.
I (5 rats)	Standard electrolyte deficient diet (SEDD)	14	140.2 ±4.15	5.17 ±.47	105.8 ±1.8	23.9 ±1.2	4.06 ±.41	51.4 ±1.2	1.94 ±.15	788 ±39
II (4 rats)	SEDD + 6mM. NH_4Cl daily	14	140.3 ±4.1	4.99 ±.26	104.1 ±2.5	17.8 ±3.3	3.43 ±.51	47.3 ±.8	5.98 ±.26	3230 ±47
III (4 rats)	SEDD + 6mM. NH_4Cl + 6mM. KCl daily	14	143.7 ±2.2	8.60 ±.50	103.7 ±1.3	17.7 ±3.7	1.43 ±.53	50.0 ±4.9	5.53 ±.26	3131 ±138
IV (5 rats)	SEDD + 2mM. NH_4Cl daily	14		4.64 ±.40		24.0 ±1.2			2.80 ±.37	1327 ±102
V (6 rats)	SEDD + 6mM. $NaHCO_3$ + 1mM. KCl daily	14		4.84 ±.38		25.2 ±1.1			.04 ±.01	508 ±42

tion of strong acids apparently augments ammonia excretion by accelerating the rate at which ammonia is formed within the tubular cell.

Since the administration of NH_4Cl produces a shift of potassium out of the cells and subsequent loss of potassium into the urine (1) and may produce a systemic acidosis, at least three changes in the internal environment might have been causally related to the activation of renal glutaminase: 1) Intracellular potassium deficiency; 2) extracellular acidosis; and 3) changes in intracellular acid-base equilibrium. The cellular content of potassium, as reflected by the potassium content of skeletal muscle, does not appear to be responsible for glutaminase adaptation, since the administration of supplemental potassium corrected the muscle deficit in three out of four rats in the present study and in all rats in a study reported elsewhere (16) (in which Diamox® resulted in glutaminase adaptation), yet prevented neither the rise in ammonia excretion nor the activation of renal glutaminase. However, since it has been demonstrated (17) that during the development of potassium deficiency the cellular content of different tissues may vary widely, the possibility remains that the potassium content of the renal tubular cells may be diminished even when muscle potassium is normal. Such a possibility was not excluded by the present study, since kidneys were not analyzed for their potassium content. Although acidosis of the extracellular fluid resulting from large acid loads (6 mM NH_4Cl) might be implicated as the causative factor for the increased glutaminase activity, rats receiving smaller acid loads (2 mM NH_4Cl) had increased glutaminase activity in the absence of a systemic acidosis. Moreover, other experiments from this laboratory on animals receiving DOCA and high sodium, low potassium diets have demonstrated a similar rise in glutaminase activity in the presence of a severe alkalosis (18). Therefore, no constant relationship between the HCO_3^- content (and probably the pH as well) of the extracellular fluid and glutaminase activity can be established. Changes in intracellular acid-base equilibrium are more difficult to analyze, since there is no satisfactory method for measuring the H^+ content of the tubular cells. A fall in intracellular pH, resulting from an acid load, might be a factor in the activation of renal glutaminase. However, *in vitro* experiments have shown the pH for optimum glutaminase activity to be approximately 8.0 (19). Nevertheless, Robinson (20) has recently demonstrated that the maximum rate of ammonia forma-

tion by kidney slices is attained at pH 5.5. This suggests either that the optimum pH *in vivo* differs radically from the optimum pH *in vitro*, or else that intracellular acidosis stimulates the formation of increased amounts of ammonia producing enzymes. While such *in vitro* data indicate that intracellular pH could modify enzymatic activity, the absence of any information of either the actual pH of the tubular cell or of the impact of pH on the integrated *in vivo* operation of the ammonia producing enzymes renders any inference from *in vitro* experiments to *in vivo* operation highly tenuous at best. The factors, therefore, which are responsible for the activation of renal glutaminase during ammonium chloride acidosis could not be identified. The present experiments indicate only that glutaminase adaptation is proportional to the magnitude of the acid load, but independent of the concentration of serum bicarbonate and the potassium content of cells (as reflected by muscle potassium).

Although the excellent correlation between ammonia excretion and glutaminase activity during NH_4Cl loading strongly suggests that the rate of ammonia excretion is, in part, regulated by the rate of *production* of ammonia within the tubular cell, the manner in which the ammonia thus produced is *transported* into the tubular urine is not precisely clear. Two theories have been formulated: 1) Free molecular ammonia (NH_3) may enter the tubular urine by a process of passive diffusion (4–8); 2) ammonium ions (NH_4^+) within the tubular cell may be transported into the urine by an active process of ion exchange for tubular Na^+ (16, 21). These theories are illustrated in the two model cells of Figure 4.

According to the theory of passive diffusion, the rate of ammonia transport is regulated by the H^+ concentration of the tubular urine, the H^+ promoting the conversion of NH_3 to NH_4^+, thereby establishing a concentration gradient favoring continued passive diffusion of NH_3 out of the tubular

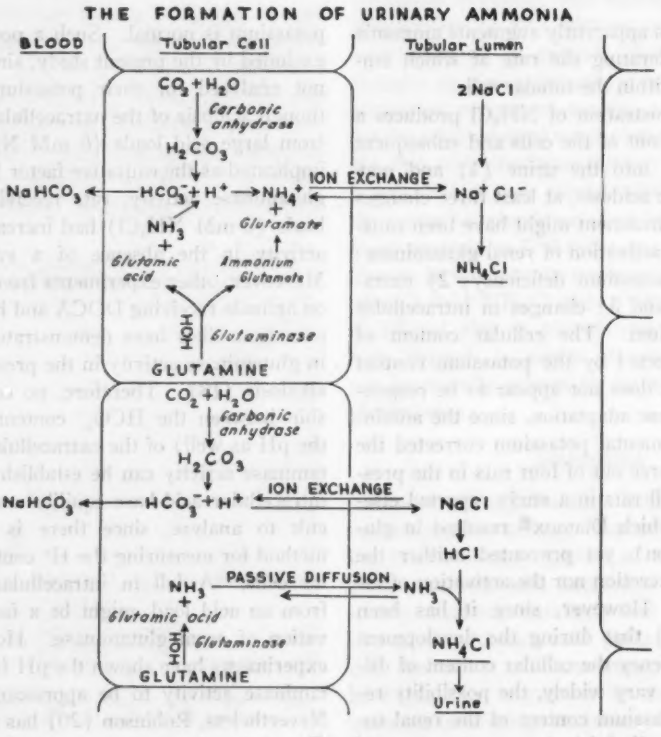

FIG. 4

cell. The data on which this theory is based have been assembled principally during acute experiments of less than 24 hours' duration (4–8). During such short intervals, major changes in the activity of the ammonia producing enzymes—judging from the data in Figure 1—do not occur, and ammonia excretion seems closely correlated with urine pH. However, during chronic acidosis, dogs excrete more ammonia at any given urine pH following an infusion of acid than do previously normal dogs subjected to the same acute acid load (5). Moreover, in the present study, with rats given varying amounts of NH_4Cl for a prolonged period of time, variations in ammonia excretion could not be attributed to any changes in urine pH. Since the rate of ammonia excretion in chronically acidotic rats varies in direct proportion to renal glutaminase activity, it is possible that accelerated formation of NH_3 within the cell may be sufficient to maintain a concentration gradient favorable for increased passive diffusion into the tubular lumen at levels of urine pH which, in acute acidosis (when the ammonia producing enzyme systems are not activated), are associated with much less ammonia excretion. The effect of urine pH on ammonia excretion can be evaluated properly, therefore, only when the rate of ammonia production can be estimated. In order to explore this relationship, ammonia excretion and glutaminase activity were determined in rats whose urine pH was elevated either by the administration of $NaHCO_3$ or Diamox® (Figure 3). These rats excreted less ammonia per unit enzyme than did rats with acid urines. The apparent discrepancy between ammonia excretion and glutaminase activity in these rats may be linked with the elevated urine pH, which could have repressed some passive diffusion of NH_3 out of the tubular cell. If passive diffusion is accepted as the primary means by which ammonia is transported into the urine it is necessary, therefore, to postulate that both the rate of ammonia formation within the cell and the pH of the tubular urine influence the concentration gradient of NH_3 across the cell membrane. This would mean, as Pitts suggested earlier (5), that the rate of ammonia excretion is pH dependent *at any given level of enzymatic activity.*

Since approximately 99 per cent of the ammonia present in the cell at physiologic pH is in the form of ammonium ion,[4] an alternative hypothesis explains the transport of NH_3 into the tubular urine on the basis of an active process of ion exchange, Na^+ of the tubular urine exchanging for NH_4^+ of the tubular cell. Although the accelerated excretion of ammonia under conditions unfavorable to passive diffusion (*i.e.*, increased urine pH) in animals receiving Diamox® (16) is highly suggestive that a mechanism of active ion exchange may be operative, sufficient data comparing urine pH and ammonia excretion with the level of enzymatic activity are not available to validate either the theory of passive diffusion or the theory of active ion exchange.

SUMMARY

1. The administration to rats of a constant NH_4Cl load resulted in a progressive, stepwise increase to a plateau in the activity of renal glutaminase, which was closely paralleled by a similar increase in ammonia excretion.

2. With the administration of different NH_4Cl loads, maximum ammonia excretion rose in direct proportion to increased glutaminase activity.

3. The adaptation of renal glutaminase was proportional to the administered acid load, but independent of the concentration of serum bicarbonate and the content of intracellular potassium.

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[4] The pK for the reaction $NH_4^+ \rightleftharpoons NH_3 + H^+$ is 9.4 (13). Therefore, in simple solution at pH 7.4 the fraction dissociated (2) can be estimated from the equation $7.4 = 9.4 + \log 2/1–2$. Since α is 0.01, 99 per cent of the total ammonia within the cell exists as NH_4^+.

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AMINOTRIPEPTIDASE CONTENT OF SYNOVIAL FLUID IN ARTHRITIC DISEASES [1]

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When an inflammatory reaction is present in the lining membrane of a fluid-containing body space, the dissolution of the inflammatory cells present in the membrane and fluid releases into the effusion the enzymes contained in these cells. The concentration of enzyme present in the fluid at any given time should be related to the degree of inflammation and might, therefore, serve as a measure of this change. The requirements for the enzyme to be chosen to measure inflammation in this way are that it should be stable, that its activity should be easily determined, and that it should be present in high concentration in leucocytes.

Aminotripeptidase is an enzyme which has been shown by Ellis and Fruton, and by Smith, to be widely distributed in tissues and to be especially richly concentrated in leucocytes (1, 2). It is stable and may be determined by measuring the rate of hydrolysis of an aminotripeptide, such as glycylglycylglycine.

Aminotripeptidase has been shown to be present in human serum by Grassmann and Heyde (3). Its concentration shows little variation in the serum of normal human adults, but is increased in certain pathological states (4), including bone fracture (5). Schwartz and Engel (6) observed moderately increased levels of aminotripeptidase activity in the serum of patients with rheumatic fever, which were reduced on administration of corticotropin.

The degree of synovial inflammation is usually measured by the leucocyte content of the synovial fluid. Leucocyte counts in synovial effusions, however, are variable and often rendered inaccurate by the presence of particulate matter. A more accurate and consistent measure of synovial inflammation is, therefore, desirable and a previous report by Ziff, Scull, Ford, McEwen, and Bunim (7) has indicated that aminotripeptidase activity may provide such a measure. The relatively high level of this enzyme in synovial effusions of patients with rheumatoid arthritis was demonstrated, and it was shown also that a decrease in the concentration of the enzyme, following intra-articular injection of hydrocortisone, was associated with improvement in the clinical status of joints and with a rise in the viscosity of the synovial fluid.

The purpose of the present investigation was to compare the aminotripeptidase levels in effusions from patients with a variety of arthritic diseases, in order to determine whether there was a relationship between the enzyme level and the severity of the inflammatory process, "severe" in this connection implying a profound change in the synovial membrane, rather than an acute but transient effect. If there were such a relationship, it would presumably be possible to separate arthritides into two extreme classes with regard to enzyme level:

1. High level: Rheumatoid arthritis,—profound inflammation and destruction.

2. Low level: Degenerative joint disease,—essentially non-inflammatory.

It would be expected, further, that there would be an intermediate or third group, comprising such diseases as rheumatic fever, gonococcal arthritis (with sterile joint effusions), Reiter's syndrome and gout; but heterogeneity would be anticipated in this group because gout and gonococcal arthritis, for example, may be markedly destructive.

Other questions to be explored were:

1. The possible value of the enzyme level in differential diagnosis.

2. The relationship of enzyme level to the leucocyte count in the synovial effusion.

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3. The comparison of enzyme levels in the synovial fluid and the blood serum.

METHODS

A total of 98 patients were studied (all the available patients with effusions of the knee during the period of the investigation)—31 with rheumatoid arthritis of the peripheral type, 17 with acute rheumatic fever (all above 12 years of age), 11 with degenerative joint disease, 2 with traumatic arthritis, 5 with gonococcal arthritis (all effusions of the sterile type), 16 with gout, and 16 with miscellaneous arthritides. One hundred and twelve synovial fluid samples were analyzed for enzyme concentration.

All samples were aspirated from knee joints using ethyl chloride anesthesia, since it has been shown (8) that procaine is a potent inhibitor of aminotripeptidase. The fluids were promptly centrifuged at 3000 r.p.m. for 20 minutes. This procedure removed the cells entirely except in very viscous samples in which, however, the cell counts were uniformly low even before centrifugation.

Aminotripeptidase was determined in synovial fluid, and in a number of instances in the serum, by the titrimetric method of Grassmann and Heyde, using thymolphthalein as indicator (9) usually within several hours of aspiration, on centrifuged, unhemolyzed specimens of synovial fluid. The substrate was glycylglycylglycine (obtained from Hoffman LaRoche, Inc., Nutley, N. J.). One-half milliliter of synovial fluid, 1.5 ml. of 0.02M veronal buffer at pH 7.8, and 0.5 ml. of 0.25M glycylglycylglycine solution, neutralized to pH 7.8, were incubated in a shaking water bath at 37° C. Two-tenth milliliter samples were removed from the mixture at intervals, and titrated with 0.015 N potassium hydroxide in 90 per cent ethanol, with five drops of thymolphthalein as indicator. Depending on the rate of hydrolysis, samples were removed at from 15 to 90-minute intervals. Five points, obtained from duplicate measurements in each case, were plotted for each determination and the per cent hydrolysis per hour was calculated from the straight line plot so obtained. Repeated determinations showed no fall in tripeptidase activity of synovial fluid stored in the refrigerator over a period of weeks. Results are given as percentage hydrolysis of glycylglycylglycine per hour.

Total leucocyte counts were done immediately after aspiration on fluid diluted twenty times with saline, and differential counts were made on stained smears of the sediment in the usual manner. Relative viscosity was measured with an Ostwald viscosimeter.

RESULTS

Preliminary impressions

In Figure 1 the distribution of aminotripeptidase activity of the entire group of 112 samples is given according to disease. For five of the principal diseases these raw data showed the following ranges in enzyme level (per cent hydrolysis per hour; number of samples in parentheses): Degenerative joint disease (13), 1.4 to 6.9; rheumatic fever (17), 3.6 to 12.2; rheumatoid arthritis (42), 3.3 to 56.3; gonococcal arthritis (5), 5.7 to 13.0; gout (18), 3.3 to 46.2.

The relatively low values observed in degenerative joint disease, rheumatic fever, gonococcal arthritis and most of the miscellaneous arthritides are in marked contrast to the relatively high and widely scattered values found in rheumatoid arthritis and, to a lesser extent, in gout.

Of eight values in the rheumatoid group falling below the 10 per cent level, three were obtained on effusions from patients with acute rheumatoid arthritis, two were from joints with osteoarthritic changes secondary to long-established rheumatoid arthritis, and two were from patients with juvenile rheumatoid arthritis. Of the latter, one patient was studied a year later and the aminotripeptidase activity had risen from 4.0 to 12.4 per cent.

In the miscellaneous group, the highest value, 42.0 per cent, was found in a patient with disseminated lupus erythematosus. The diagnosis was based on the findings of L.E. cells in the peripheral blood and evidence of renal disease; but the joints showed chronic deforming changes identical with those of rheumatoid arthritis.

Analysis of data

The main analyses were confined to cases with the following characteristics:

1. Those with dependable information regarding the duration of symptoms, prior to investigation, in the joint examined.

2. Those with a definite record that, during the period immediately preceding the investigation, no treatment (except analgesics in a few instances) had been administered.

3. Those of age 18 and over.

The numbers of patients whose data qualified for the analyses were: degenerative joint disease, 8; rheumatoid arthritis, 14; rheumatic fever, 6; gout, 13; gonococcal arthritis, 4; Reiters's syndrome, 3. Standard statistical methods (analysis of variance and covariance, regression, and correlation) were employed, and the 5 per cent level

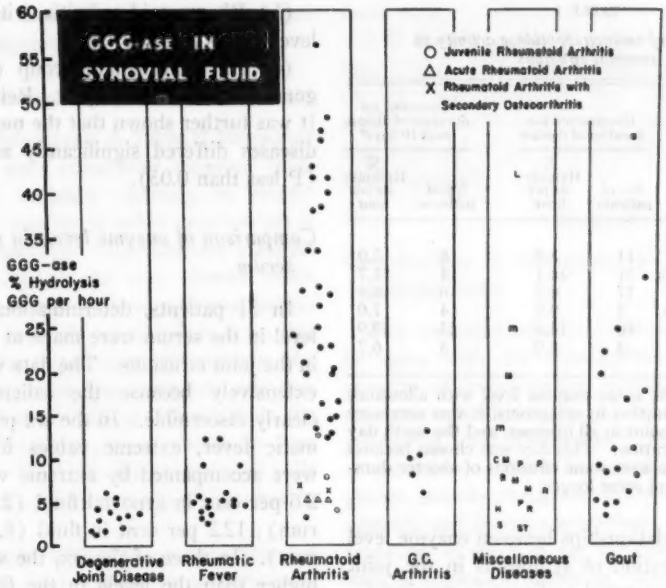

FIG. 1. DISTRIBUTION OF AMINOTRIPEPTIDASE LEVELS OF THE SYNOVIAL FLUID IN A NUMBER OF ARTHRITIC DISEASES

L = Disseminated lupus erythematosus
M = Marie-Strumpell disease
m = Meningococcemia (fluids sterile)
C = Coccidioidomycosis
P = Psoriatic arthropathy
R = Reiter's syndrome
H = Henoch-Schoenlein purpura
S = Subacute bacterial endocarditis
St = Streptococcal pneumonia

was used as the minimum criterion for significance, *i.e.*, P (the probability of occurrence if chance alone were operating) had to be less than 0.05 before a verdict of significance was given. (During the analysis it was found appropriate, for reasons that need not be discussed here, to express both the enzyme level and the duration of joint symptoms as logarithms, but the numerical values, given later, are in the original units, per cent hydrolysis per hour, obtained by reconversion). The results concerning the six diseases just enumerated (see Table I) can be summarized thus:

1. There was sufficient evidence of a positive correlation between enzyme level and the previous duration of symptoms in the joint aspirated to make it very likely that some of the variation in enzyme level between patients was associated with a difference in duration. This association could be due to one or both of two causes:

(a) Increase of enzyme concentration as the disease progressed.

(b) The longer duration of effusion in joints that were from the beginning more severely affected and, therefore, had a higher enzyme concentration throughout.

This second possibility could not be excluded, but the data did not suggest that it was a major factor, and, whatever the cause of the correlation, it was necessary to correct for it in order to obtain a reliable comparison between the mean enzyme levels in the various diseases.

2. There was no suggestion of sex or age difference in enzyme level, and, therefore, these characteristics were disregarded in further analysis.

TABLE I

Mean levels of aminotripeptidase activity in synovial effusions

Disease	Uncorrected for duration of disease		Corrected for duration of disease as at 10 days*	
	No. of patients	% Hydrolysis per hour	No. of patients	% Hydrolysis per hour
Degenerative joint disease	11	3.6	8	2.0
Rheumatoid arthritis	31	24.1	14	14.7
Rheumatic fever	17	6.2	6	4.4
Gonococcal arthritis	5	9.6	4	7.0
Gout	16	14.4	13	10.9
Reiter's syndrome	3	6.0	3	6.2

* In estimating the mean enzyme level with allowance for the effects of duration in symptoms, it was necessary to choose the same point in all diseases, and the tenth day was taken for illustration. This day was chosen because in each disease there were some effusions of shorter duration than 10 days and some longer.

3. When the relationships between enzyme level and previous duration of symptoms in the joint examined were expressed as linear regression coefficients (*i.e.*, as the slopes of the best-fitting straight lines) no significant differences of slope were found among the six diseases.

4. In Figure 1 there appears to be a striking contrast in interpatient variation between such diseases as rheumatoid arthritis and rheumatic fever. Since such differences in variation are often correlated with differences in average values, the logarithms of the experimental values were used in comparing interpatient variation, and it was then found that this variation did not differ significantly among the diseases. The contrasts in Figure 1 can, therefore, be adequately accounted for by differences in general level in the different diseases—the higher the general or average level, the greater the interpatient variation.

5. Even after allowance for the enzyme-duration relationship (Table I), there was a highly significant difference in the mean enzyme levels among the six diseases (P far less than 0.001), and this agrees with the initial hypothesis regarding the grouping of diseases, as follows:

(a) Degenerative joint disease, with lowest enzyme level. (The two cases of traumatic arthritis, although insufficient to include in the full analysis, resembled degenerative joint disease in enzyme level.)

(b) Rheumatoid arthritis, with highest enzyme level.

(c) An intermediate group (rheumatic fever, gonococcal arthritis, gout, Reiter's syndrome). It was further shown that the mean levels in these diseases differed significantly among themselves (P less than 0.05).

Comparison of enzyme levels in synovial fluid and serum

In 31 patients, determinations of the enzyme level in the serum were made at the same time as in the joint effusions. The data were not analyzed extensively because the salient features were clearly discernible. In the six patients with rheumatic fever, extreme values for synovial fluid were accompanied by extreme values for serum: 2.6 per cent in synovial fluid (2.3 per cent in serum); 12.2 per cent in fluid (8.0 per cent in serum). In three of the six, the serum levels were higher than the levels in the fluid, in the other three the difference was reversed. Two patients with degenerative joint disease gave the following readings in fluid and serum: 3.5 per cent (3.8 per cent in serum); 1.7 per cent (2.7 per cent in serum). These data suggest that in rheumatic fever and degenerative joint disease the synovial inflammation has little influence in increasing the tripeptidase level of the synovial fluid above the serum.

In the 14 patients with rheumatoid arthritis, the synovial fluid levels ranged from 5.3 per cent to 48.5 per cent; the serum levels from 2.4 per cent to 7.6 per cent. In all these patients the level in fluid exceeded the serum level, the contrasts ranging from 5.3 per cent in fluid (3.7 per cent in serum) to 46.0 per cent in fluid (3.0 per cent in serum).

In the nine patients with gout the fluid levels ranged from 3.3 per cent to 19.5 per cent, the serum levels from 1.7 per cent to 6.2 per cent; and the fluid levels exceeded the serum levels in all except one instance (fluid, 4.7 per cent, serum, 4.8 per cent).

Two noteworthy features in the above data are the general similarity of serum enzyme levels in the various diseases, and the discrepancy between serum levels and the levels in the synovial fluid in rheumatoid arthritis and gout, in contrast to the

similarity of these levels in rheumatic fever and the two cases of degenerative joint disease.

Relationship of enzyme level to leucocyte count

In the experiments shown in Table II leucocytes and erythrocytes were completely removed from synovial fluid of ordinary viscosity by centrifugation, and in a number of other experiments it was shown that leucocytes were likewise successfully removed except in the very viscous fluids where the white counts and tripeptidase values were consistently low. It would follow from this that, at least in the usual range of viscosity, the tripeptidase activity that was measured was due largely or entirely to the enzyme dissolved in the synovial fluid. Moreover, as is shown by two of the samples in Table II, the presence of cells does not necessarily cause a marked increase in the enzyme level.

In the 45 patients whose data were subjected to detailed analysis there was only a moderate degree of correlation between the leucocyte counts and the enzyme levels, and the correlation did not differ significantly between the diseases. The average coefficients of correlation with enzyme level were + 0.38 for the total white count and + 0.43 for the polymorphonuclear count (P less than 0.02). The scatter diagrams (Figures 2 and 3) of total leucocyte and polymorphonuclear counts of freshly aspirated synovial fluid for the entire group of patients illustrate correlations of this magnitude.

DISCUSSION

Classification of arthritides by characteristics of synovial fluids

The aminotripeptidase levels have been shown to agree with the classification of diseases by severity of inflammation postulated at the outset: rheumatoid arthritis at the one extreme, degenerative joint disease (and traumatic arthritis) at the other, with an intermediate heterogeneous group, notably rheumatic fever, gout, gonococcal arthritis and Reiter's syndrome. It is of interest, however, to see how far the enzyme levels agree with a somewhat different classification, namely that of Ropes and Bauer (10) which is based on characteristics of the synovial fluid other than enzyme levels. In Group I of Ropes and Bauer are placed degenerative joint disease and traumatic

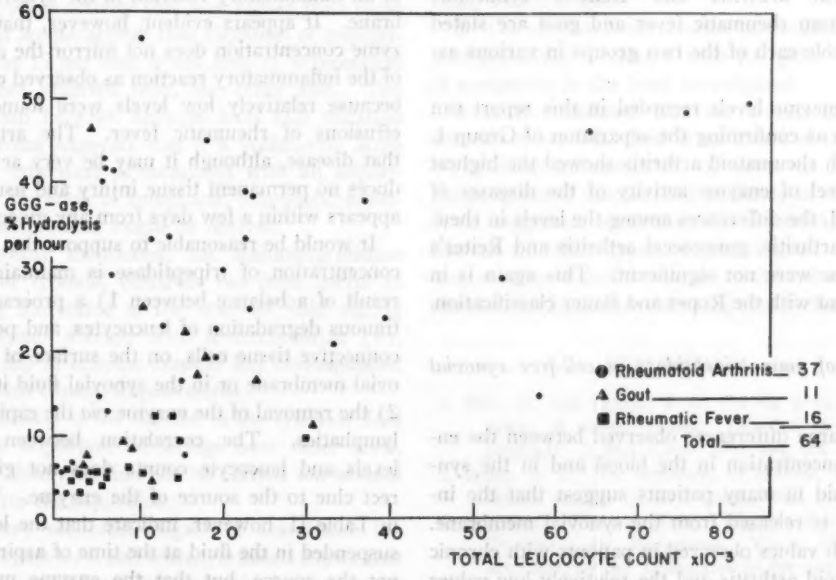

FIG. 2. AMINOTRIPEPTIDASE LEVELS PLOTTED AGAINST TOTAL LEUCOCYTE COUNT OF THE SYNOVIAL FLUID

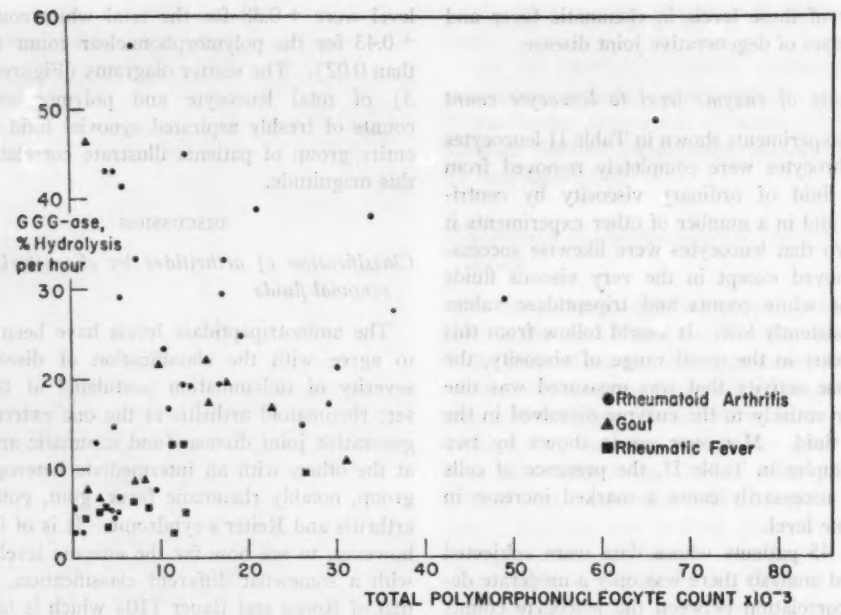

FIG. 3. AMINOTRIPEPTIDASE LEVELS PLOTTED AGAINST POLYMORPHONUCLEOCYTE COUNT OF THE SYNOVIAL FLUID

arthritis. In Group II occur rheumatoid arthritis, gonococcal arthritis and Reiter's syndrome. Fluids from rheumatic fever and gout are stated to resemble each of the two groups in various aspects.

The enzyme levels recorded in this report can be taken as confirming the separation of Group I. Although rheumatoid arthritis showed the highest mean level of enzyme activity of the diseases of Group II, the differences among the levels in rheumatoid arthritis, gonococcal arthritis and Reiter's syndrome were not significant. This again is in agreement with the Ropes and Bauer classification.

Source of aminotripeptidase in cell-free synovial fluid.

The large differences observed between the enzyme concentration in the blood and in the synovial fluid in many patients suggest that the increment is released from the synovial membrane. The high values observed in patients with chronic rheumatoid arthritis and the relatively low values in the presence of degenerative joint disease suggest that the increase is dependent on the presence of an inflammatory reaction in the synovial membrane. It appears evident, however, that the enzyme concentration does not mirror the acuteness of the inflammatory reaction as observed clinically, because relatively low levels were found in the effusions of rheumatic fever. The arthritis of that disease, although it may be very acute, produces no permanent tissue injury and usually disappears within a few days from any given joint.

It would be reasonable to suppose that a steady concentration of tripeptidase is maintained as a result of a balance between 1) a process of continuous degradation of leucocytes, and perhaps of connective tissue cells, on the surface of the synovial membrane or in the synovial fluid itself, and 2) the removal of the enzyme *via* the capillaries or lymphatics. The correlation between enzyme levels and leucocyte counts does not give a direct clue to the source of the enzyme. The data in Table II, however, indicate that the leucocytes suspended in the fluid at the time of aspiration are not the source, but that the enzyme must have been released prior to the aspiration.

TABLE II

Cell counts and aminotripeptidase activity of centrifuged and uncentrifuged specimens of synovial fluid

Patient	Sample	Relative viscosity	Cell count per mm³ RBC	Cell count per mm³ WBC	Aminotripeptidase activity % hydrolysis per hour
D. K.	Uncent.	—	14,500	8,700	11.6
	Cent.	—	0	0	10.5
M. F. (Right knee)	Uncent.	—	25,700	10,950	37.6
	Cent.	6.5	0	0	36.5
M. F. (Left knee)	Uncent.	—	22,600	9,300	36.7
	Cent.	8.8	0	0	30.5
D. L.	Uncent.	—	94,000	25,300	79.0
	Cent.	4.7	0	0	49.0

Practical application of enzyme determinations

As with other laboratory indices of the synovial fluid, there is an overlapping of enzyme values in the different diseases (Figure 1) which renders definitive diagnosis from the synovial fluid difficult. Values above 6.9 per cent, however, were not seen in degenerative joint disease, nor were values above 12.2 per cent found in rheumatic fever. Since approximately three-fourths of the patients with rheumatoid arthritis had effusions with peptidase activity that was higher than these figures, it would appear that elevation of tripeptidase above 12 per cent would help to differentiate rheumatoid arthritis from the other two. The relationship between enzyme level and duration of symptoms suggests that if a sufficiently large body of data were accumulated with an adjustment for duration of symptoms, diagnostic standards of some value might be derived.

As already mentioned, the aminotripeptidase activity of synovial effusions has been useful in following the status of the synovial fluid during intra-articular therapy with hydrocortisone (7), and the fall in enzyme content has corresponded closely with clinical improvement.

The stability of the enzyme in the synovial fluid offers an important advantage over other tests, since the determination can be carried out several weeks after the aspiration.

The very moderate degree of correlation between enzyme level and leucocyte count shows that the count would be of little value in estimating the enzyme level.

Finally, it should be pointed out that the possible utility of aminotripeptidase content in relation to assessment of inflammation is not restricted to synovial fluid. The authors have demonstrated activity of the same enzyme in empyema fluid and in purulent sputum, and it is likely that it may be found in almost any effusion associated with inflammation.

SUMMARY

1. Using glycylglycylglycine as substrate, the aminotripeptidase content of synovial effusions was measured in 98 patients with a wide variety of arthritic diseases.

2. Arthritides were grouped according to the severity of the inflammatory process in three classes: two extremes—degenerative joint disease (non-inflammatory) and rheumatoid arthritis (profound inflammation); and an intermediate group—rheumatic fever, gonococcal arthritis (with sterile effusions), Reiter's syndrome and gout. The three groups differed significantly in mean enzyme level, with the lowest values in degenerative joint disease and the highest in rheumatoid arthritis. The levels in the intermediate group, also, differed significantly, the highest level being found in gout.

3. The enzyme concentration in various diseases was positively correlated with the prior duration of symptoms in the joint investigated.

4. It is concluded that the aminotripeptidase content of synovial fluid is an index of the status of synovial inflammation, of value in following the progress of disease, and probably in diagnosis. It is suggested, also, that the aminotripeptidase content of other types of pathological effusions may serve as a useful measure of the inflammatory reaction in the tissues enclosing these effusions.

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THE DISTRIBUTION AND EXCRETION OF S^{35}-LABELED SULFO-BROMOPHTHALEIN-SODIUM ADMINISTERED TO DOGS BY CONTINUOUS INFUSION [1]

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It has been known for some time that continuous intravenous infusion of sulfo-bromophthalein-sodium (referred to below as BSP) in dogs as well as in man results eventually in a steady state in which blood and bile concentrations, and usually the rate of biliary excretion of the dye, remain constant (1). Bradley and co-workers have pointed out that such a steady state can only be achieved at infusion rates resulting in blood levels of BSP lower than a certain liminal value (20 mg. per L. in man [2] or 40 mg. per L. according to other workers [3]). The present authors (4) have pointed out in a previous communication that during such steady state conditions, the rate of biliary excretion of the dye is consistently below the rate of infusion, indicating that considerable amounts of sulfo-bromophthalein-sodium are being stored in the body. This is in accord with observations by Cantarow and Wirts (5) who showed that, following the administration of single doses of sulfo-bromophthalein-sodium, dye excretion into bile lagged by a considerable period of time behind the disappearance of most of the dye from the blood stream; they concluded that this period must represent storage of the dye.

The actual sites of storage of this "lost BSP" could not be determined with certainty in either of these investigations; indications were obtained that a considerable amount of dye is retained in the liver prior to excretion. Further investigation of this point seemed indicated for two reasons: First, application of BSP to clinical studies of hepatic blood flow depend, to a considerable extent, upon assumptions regarding the storage of the dye in tissues supplied by the splanchnic circulation; second, BSP is a most useful prototype substance for theoretical studies of bile formation and bile secretion, and knowledge of its actual sites of deposition is essential if serious errors of interpretation are to be avoided in this connection.

The preparation of S^{35}-labeled BSP (6) provides a tool which permits a direct approach to these questions. The present communication deals with the application of S^{35}-labeled BSP to the study of the distribution of BSP when administered to dogs by continuous intravenous infusion.

MATERIALS AND METHODS

Mongrel dogs under sodium pentobarbital anesthesia were studied after cholecystectomy and preparation of common bile duct fistulae. When desired, liver injury was induced by means of CCl_4 administered by stomach tube (0.5 cc. per Kg., 72 and again 24 hours before operation). BSP[4] was administered intravenously by means of a continuous infusion pump. For details of animal selection and maintenance, of induction of liver injury and surgery see (4).

S^{35}-labeled BSP was prepared by sulfonation of tetrabromphenolphthalein and purification of the product by chromatography and recrystallization (6). Colorimetric analyses for BSP were undertaken in the cases of the blood and bile samples only. The procedure employed, a modification of that described in (7), cf. also (8), consisted of diluting the samples with water, then acetone, in such fashion as to attain final protein concentrations between 0.8 and 1.2 per cent, and acetone concentrations of 60 per cent by volume. Blood plasma samples were then stored in the refrigerator for one hour and the protein precipitate removed by centrifugation; bile samples could be used without such intermediate treatment. Aliquots of the solutions were collected, 0.1 cc. of 10 per cent NaOH added to 2 cc. of solution, and suitable amounts transferred to the 10 mm. plunger microcell of

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[4] BSP and the precursor for S^{35} BSP were obtained through the courtesy of Dr. H. B. Dunning, Hynson, Westcott and Dunning, Inc., Baltimore, Maryland.

an Evelyn photoelectric colorimeter. BSP concentrations were determined from the optical densities (580 mμ filter) by means of a calibration curve. In the presence of plasma protein, BSP recoveries of 90 to 100 per cent can be attained readily, the average loss in a series of experiments with different plasma samples being 3.5 per cent over the range of BSP concentrations of interest here. Table 1 shows a typical experiment in which BSP loss in the precipitate is determined at three levels of BSP concentration covering the range of values observed in the present series. In calculating blood BSP concentrations one may either use an aqueous solution calibration curve and correct for BSP loss using the above values (cf. Table 1), or, where extreme accuracy is essential, set up individual plasma calibration curves for each experiment. For the present series the former procedure was resorted to. In the case of the bile samples, preparation for colorimetry does not involve any material loss, so that equivalent BSP concentrations can be calculated directly from the optical density. The addition of bile in the amounts here involved to solutions of BSP does not alter the absorption coefficient of this dye at 580 mμ.

Analyses for S^{35} content were carried out on tissue homogenates as well as on blood and bile specimens. Blood and bile S^{35} determinations were carried out using the same solutions as employed for colorimetry. One- to two-tenths cc. of these were plated directly on tissue-paper-covered aluminum planchets, dried, and counted using a 1.4 mg. per cm^2 end-window Geiger-Muller Counter. The count rate was converted to equivalent BSP concentrations by comparison with a calibration curve using identically prepared planchets containing known amounts of the S^{35} BSP preparation used for the particular ex-

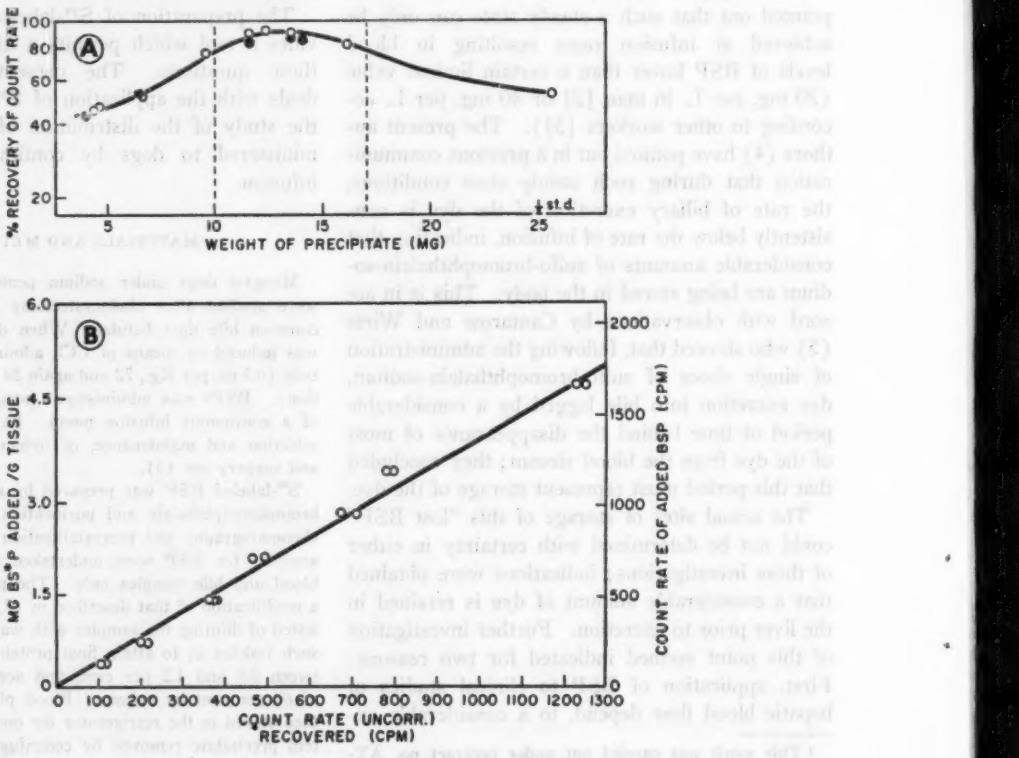

FIG. 1. S^{35} RECOVERY IN TISSUE ANALYSES

a. Relation between BSP-S^{35} recovered after addition of constant amounts of dye to varying amounts of dog liver, and weight of precipitate collected. Region between dashed lines represents conditions acceptable for analytical purposes.

b. Relation between amount of BSP-S^{35} added to a constant amount of dog liver, and S^{35} recovery.

TABLE 1

RECOVERY OF BSP FROM DOG BLOOD PLASMA AFTER ACETONE DEPROTEINIZATION

CONCENTRATION OF BSP IN PLASMA -- MG/L	CONCENTRATION AFTER ACETONE DEPROTEINIZATION*	% RECOVERY
15.8	15.2	96.4
37.4	36.2	96.8
68.4	65.0	95.3

* After correction for dilution, and using the proper acetone-saline-BSP calibration curve.

periment. Recovery of added BSP-S^{35} from blood plasma is controlled by the same factors which control recovery of colorimetric BSP; recoveries, therefore, are the same as shown in Table 1. Bile samples were prepared without material loss; S^{35} recoveries, after correction for self absorption, are 100 ± 1.0 per cent. Specific activity determinations are independent of BSP recovery in any case, since the same final solution was used for colorimetry and for radioassay. Standard deviations for specific activity determinations are about ± 2 per cent provided duplicate determinations are employed.

S^{35} in the various tissue homogenates was determined by the alkaline permanganate combustion procedure described in (9), followed by collection of the precipitates on filter paper in a precipitation apparatus, drying to constant weight, and counting as above. Recoveries of count rate are influenced by two factors varying in opposite direction with the weight of precipitate collected: Incomplete recovery of precipitate on the filters, and self absorption. Figure 1a shows a typical calibration curve obtained by adding the same, known, amount of S^{35} BSP to varying amounts of liver tissue. Again, using varying amounts of BSP with a constant amount of tissue the count rate obtained is proportional to the amount added (Figure 1b). Assuming such a linear relation, the standard deviation of individual points from the least square line is 10.7 per cent. Table 2 shows the recovery of known amounts of BSP added to different tissues and calculated from the actual count rates using the calibration curve of Figure 1a. Since recovery does not change excessively with precipitate weights in the range of 10 to 17 mg., determinations of tissue BSP-S^{35} should be carried out within this range of sample sizes. Experience has shown that the nature of the analytical procedure employed here is such that consistent results can be expected if, but only if, all details of the entire procedure are carried out identically in the minutest degree. Best results were obtained by carrying an entire series of determinations, including unknown samples and samples set up for determination of the calibration curve, through the entire series of steps in parallel and at the same time. Each dog experiment reported below was treated in this fashion and the estimates of standard deviation for each point derived from the above data apply. Using triplicate samples, each individual tissue concentration has a standard deviation of the mean of ± 6.7 per cent.

RESULTS

A. Color and activity relations in blood and bile

After a period of one to two hours of constant infusion of S^{35}-labeled BSP, in all dogs blood levels of the dye were attained which varied by no more than ± 10 per cent with continued infusion at the same rate. In normal dogs, bile levels also had become steady (± 8 per cent) at this time; in CCl_4 treated dogs, three hours of constant infusion might be required before bile levels reach a plateau. Figure 2 shows representative experiments indicating the course of blood and bile BSP concentrations as calculated from S^{35} content, as well as the ratio of BSP—calculated from S^{35} content—to BSP determined colorimetrically. Relevant data concerning this ratio, and its components, during the steady state in a series of experiments, are summarized in Table 3. In all normal dogs the S^{35} content of the blood plasma agreed within the limits of experimental error with values predicted on the basis of the colorimetric determinations of BSP in the same samples. $\left(\frac{\text{BSP-}S^{35}\text{ Activity}}{\text{BSP color}}\text{ ratio equal to one.}\right)$ In bile, on the other hand, an excess of S^{35} over that which could be accounted for as colorimetrically determined BSP was found in each case. This excess was found quite variable, ranging from 15 per cent in one case to as much as 62 per cent at the other extreme. No correlation was found between

TABLE 2

RECOVERY OF BSP-S^{35} ADDED TO VARIOUS TISSUES

TISSUE	TISSUE $BS^{35}P$ CONCENTRATION -- MG/KG	BSP-S^{35} RECOVERED -- %
LIVER	(547 to 3750	97.0 ± 2.8*
SPLEEN	114 135 198	101 91.6 90.4
KIDNEY	135 135 135	92.6 79.1 83.8
MUSCLE	135 124	89.3 91.0
DUODENUM	138 138 138	78 102 100
COLON	121 132 190	104 145 110
STOMACH	128 190	104 89
MEAN ± ST. D. M. ST. D. SINGLE DETN.		95.7 ± 3.0 ± 11.7

*Values from Figure 1b; standard deviation for single determination ± 10.8.

TABLE 3
RELATIONS BETWEEN COLORIMETRICALLY DETERMINED BSP, AND S^{35} DURING THE STEADY STATE PHASE OF CONTINUOUS INFUSIONS OF S^{35} BSP IN DOGS

DOG #	RATE OF S^{35} BSP INFUSION MGM/KGM/MIN	BLOOD PLASMA		BILE			
		S^{35} CONCENTRATION (AS BSP)—MGM/I	S^{35} (AS BSP) / COLORIM. BSP	S^{35} CONCENTR. (AS BSP) GM/I	S^{35} (AS BSP) / COLORIM. BSP	RATE OF EXCRETION % OF INFUSION RATE COLORIM. BSP	S^{35}
		NORMAL DOGS					
50-26	0.076	5.3	1.00	4.4	1.51	33.0	49.8
50-21	0.085	9.9	1.01	3.0	1.35	27.6	37.0
50-24	0.085	14.5	1.03	4.6	1.62	39.2	45.0
50-5	0.150	22.0	1.00	10.2	1.18	43.2	52.1
50-6	0.183	38.0	1.05	8.8	1.18	58.8	69.2
51-4	0.196	37.6	1.02	10.6	1.15	48.7	57.0
51-9	0.196	14.9	1.06	16.9	1.58	42.4	66.2
51-7	0.236	22.4	1.01	10.5	1.26	45.8	58.0
51-3	0.261	21.1	0.99	16.6	1.54	50.7	77.0
		CCl_4 TREATED DOGS					
50-16	0.090	234.	0.95	2.9	1.00	12.1	12.1
50-25	0.112	109.	1.06	2.9	1.19	3.9	4.7
50-9	0.144	220.	0.95	5.1	1.03	11.8	12.0
51-22	0.173	102.	1.00	7.5	1.46	2.9	4.3
51-21	0.204	47.3	1.02	16.5	1.31	35.3	46.4

the magnitude of excess of S^{35} and the infusion rate.

Inert sodium sulfate was added to several samples of bile containing S^{35} BSP. Separation of this carrier sulfate by means of acetone precipitation indicated that less than 2 per cent of the S^{35} in such bile was present as inorganic sulfate. The S^{35} in bile was not extractable with ether.

B. Retention of S^{35} BSP

In spite of the fact that the biliary concentration of S^{35} was greater than could be accounted for in terms of colorimetric BSP, the rate of S^{35} excretion into bile was still found to be significantly below the rate of BSP-S^{35} infusion in all experiments (Table 3, last column). Urine formed during the infusion of BSP in these dogs contained either no detectable BSP (seven cases) or else contained small amounts of BSP (two cases), never in excess of 1.5 per cent of the total S^{35} infused. Thus, considerable amounts of BSP are being stored in the bodies of these animals even during the steady state phase. The ratio between BSP-S^{35} infused and BSP-S^{35} excreted into bile approached more closely to 1.0 at the higher rates of infusion. Since, according to Bradley (2), excessive rates of infusion result in increasingly high apparent hepatic plasma flow, *i.e.*, increasing relative importance of extrahepatic BSP extraction in hepatic blood flow determinations by the BSP method, this finding in the dogs indicates that the range of infusion rates employed here cannot have been outside the acceptable limits for such studies.

C. Distribution of BSP-S^{35} in dogs after continuous infusion to steady state

The amount of BSP-S^{35} retained in the carcasses of dogs could be calculated as the difference between the amount infused and the amount excreted into bile with a minor correction for urinary BSP-S^{35}. The distribution of the material retained was determined in four normal and two CCl_4 treated animals by direct determination of S^{35} concentrations in the tissues. The results of these analyses are presented in Table 4. The figures for individual tissue concentrations of S^{35} were converted to "estimated total fraction of BSP-S^{35} retained" by multiplication either with directly determined tissue weight (liver, kidney, intestine, urinary bladder, heart, spleen, lung, adrenal, pancreas, and brain) or with the tissue weight as estimated from mean values given in (10) (skeletal muscle, fat); these values also are included in Table 4. An over-all survey of the results obtained is presented in Table 5 which indicates that between 34 and 43 per cent of the BSP-S^{35} infused was retained in the carcasses of these animals; between 48 and 75 per cent of BSP retained was stored in the liver; the concentration of BSP in the liver was between 30 and 57 times as high as the concentration in the rest of the carcass.

The amount of BSP not retained by the liver appeared to be loosely related to plasma BSP levels: The ratio of rest carcass BSP-S^{35} concentrations to plasma BSP-S^{35} concentrations ranged from 0.39 to 0.59.

TABLE 4
THE DISTRIBUTION OF S^{35} CALCULATED AS BSP THROUGH THE BODY OF DOGS AFTER CONTINUOUS INFUSIONS OF S^{35} BSP

DOG #	51-4		51-9		51-7		51-3		51-22		51-21	
WT., SEX	10.2 KGM		9.0 KGM		7.0 KGM		10.2 KGM		13.2 KGM		8.2 KGM	
NORMAL OR CCl_4	NORMAL		NORMAL		NORMAL		NORMAL		CCl_4		CCl_4	
RATE OF INFUSION – MGM BSP/KGM/HR	0.196		0.196		0.236		0.261		0.173		0.205	
TOTAL INFUSED–MGM	625		616		396		410		530		548	
S^{35} CALC. AS BSP IN:	MGM/KGM	% TOTAL INFUSED	MGM/KGM	% TOTAL INFUSED	MGM/KGM	% TOTAL INFUSED	MGM/KGM	% TOTAL INFUSED	MGM/KGM	% TOTAL INFUSED	MGM/KGM	% TOTAL INFUSED
BLOOD PLASMA	37.6	1.7	14.9	0.6	22.4	1.2	21.1	1.5	102	7.1	47.3	1.9
LIVER	390.	21.8	360.	25.3	525.	20.0	305.	23.5	625	46.0	579.	33.0
BILE	16,680.	58.0	15,600.	66.1	10,500.	57.5	10,590.	56.8	7,460	2.9	16,500.	28.4
STOMACH	165.	3.2	12.0	0.1	30.0	0.5	84.0	2.7	—	—	—	—
SMALL INTESTINE	49.0	2.6	16.0	1.1	31.1	2.4	36.0	2.9	—	—	—	—
LARGE INTESTINE	20.1	0.2	15.0	0.2	18.9	0.5	16.0	0.2	—	—	—	—
CONTENTS SM. INTEST.	—	—	7.0	<0.1	5.0	<0.1	—	—	—	—	—	—
CONTENTS L. INTEST.	6.0	<0.1	9.0	<0.1	10.0	0.1	4.0	<0.1	—	—	—	—
KIDNEY	36.5	0.3	90.0	1.2	72.3	1.0	37.0	0.5	400.	5.3	118.	1.0
URINARY BLADDER	—	—	22.0	0.0	16.0	<0.1	—	—	—	—	—	—
URINE	NIL	0.0	320.	0.8	NIL	0.0	44.4	0.2	20.7	0.2	267.	1.7
SKEL. MUSCLE – RECT. ABD. OR QUADRUCEPS*	12.2	8.6	5.3	3.0	13.2	10.0	3.3	3.6	—	—	—	—
INTERCOSTAL	32.0	—	22.0	—	—	—	41.0	—	—	—	—	—
HEART	12.0	0.2	14.0	0.2	13.9	0.2	26.0	0.6	—	—	—	—
SPLEEN	11.1	0.1	—	—	16.0	0.1	5.7	0.1	—	—	—	—
LUNG	4.0	0.1	—	—	2.7	0.1	12.0	0.3	—	—	—	—
ADRENAL	6.0	0.0	—	—	—	—	11.0	0.0	—	—	—	—
PANCREAS	—	—	14.0	0.1	53.0	0.2	67.0	0.2	—	—	—	—
BRAIN	—	—	1.5	0.0	1.3	0.0	—	—	—	—	—	—
FAT**	(8)	(2.5)	8.5	2.3	8.2	2.7	8.4	3.9	—	—	—	—
TOTAL IN TISSUES SAMPLED	—	99.4	—	100.1	—	96.6	—	97.1	—	—	—	—

*MEAN OF QUADRUCEPS, RECTUS ABDOMINIS, OCCIPITAL AND EXTERNAL OBLIQUE MUSCLES. ESTIMATED AS 40.3% OF BODY WEIGHT
**ESTIMATED AS 19% OF BODY WEIGHT.

The detailed distribution of extrahepatic BSP can be seen from Table 4. Tissues showing relatively high concentrations of BSP compared to the plasma levels were: stomach and small intestine, pancreas, intercostal muscle, and especially kidney. The appearance of BSP in urine is sporadic; to date no way has been found of predicting the occurrence of urinary BSP. There does not appear to be any correlation with plasma levels nor with BSP-S^{35} concentrations in the kidney. The concentrations of BSP in the urine of those animals which did show urinary BSP were considerably higher than in blood plasma, thus indicating that under certain circumstances

TABLE 5
SUMMARY OF DATA REGARDING DISTRIBUTION OF INFUSED BSP S^{35} IN DOGS

DOG #	51-4	51-9	51-7	51-3	51-22	51-21
% OF INFUSED S^{35} RETAINED IN BODY	NORMALS 42.0	33.9	42.5	43.2	CCl_4 TREATED 97.1	71.6
% OF INFUSED S^{35} IN LIVER AND BILE	80.5	92.1	77.5	80.0	48.1	61.0
% OF S^{35} (RETAINED IN BODY) FOUND IN LIVER	53.2	74.7	47.5	54.1	46.5	46.4
S^{35} CONCENTRATION (AS BSP) IN BLOOD PLASMA – MGM/KGM	37.6	14.9	22.4	21.1	102.	47.3
DTO. IN CARCASS EXCLUDING LIVER (CALC.)	12.8	6.3	13.3	8.4	25.6	21.9
RATIO OF $\frac{S^{35}\text{ CONCENTRATION LIVER}}{\text{PLASMA}}$	10.4	24.2	23.5	14.5	6.2	12.2
DTO. $\frac{\text{REST CARCASS}}{\text{PLASMA}}$	0.34	0.42	0.59	0.39	0.25	0.46
DTO. $\frac{\text{LIVER}}{\text{REST CARCASS}}$	30.7	57.0	39.7	37.4	24.8	26.5

the kidney is capable of concentrating BSP. Extremely low concentrations of S^{35} were found in the brain in the two cases examined, and low concentrations were found in intestinal contents.

The largest reservoir of extrahepatic BSP appears to be the skeletal musculature. This is due primarily to the large mass represented by this tissue. Concentrations of S^{35} in various large muscles were found relatively constant and low. By contrast the respiratory muscles, in particular the intercostal muscles, tended to show high concentrations of BSP-S^{35} relative to blood plasma. Cardiac muscle took an intermediate position following more closely values found in the blood plasma. For calculation of the total amount retained in skeletal muscle the mean of concentrations in various large muscles was employed; respiratory muscles—which alone showed higher concentrations—account for no more than 5 per cent of the total muscle mass; their contribution to the extrahepatic BSP storage should be less than 1 per cent of the total infused. Finally, attention may be called to the surprisingly large contribution to the total BSP storage made by body fat; *in vitro*, BSP has been found insoluble in fat solvents at all physiological pH values. The total contribution due to fat is probably over-estimated since it is based on the figures of reference (10) which were obtained by total extraction, and thus, in all probability, include lipids extracted from tissue other than the fat depots.

D. Continued accumulation of BSP-S^{35} in depots during steady state phase

Comparison of the figures for BSP-S^{35} concentrations in respiratory muscles, on the one hand, and in the large muscles of the various dogs, on the other hand, suggests that the latter at any rate have not exhausted their capacity to take up BSP. To test this point, an experiment was conducted in which duplicate biopsy specimens of corresponding portions of the right and left rectus abdominis were taken, the first shortly after the establishment of steady blood levels, and the second two hours later. S^{35} contents of these tissue calculated as BSP were 2.75 and 2.57 mg. per Kg. after 67 minutes of infusion, and 5.07 and 6.02 mg. per Kg. after 189 minutes of infusion (Experiment 51–9). It appears, therefore, that

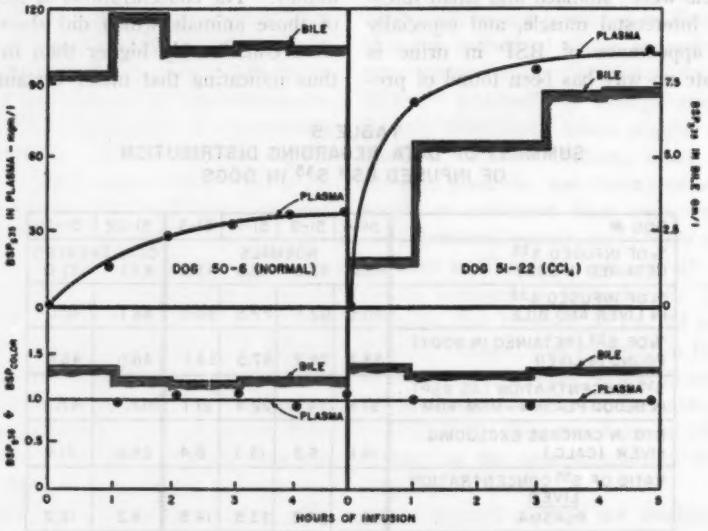

FIG. 2. CONTINUOUS INFUSION OF BSP INTO FEMORAL VEIN OF DOGS UNDER SODIUM PENTOBARBITAL ANESTHESIA

Dog 50-6, ♂ 8.2 Kg., Infusion rate 0.183 mg. per Kg. per min. Dog 51-22, ♂ 13.2 Kg., Infusion rate 0.173 mg. per Kg per min., pretreated with CCl_4.

BSP uptake, by the large skeletal muscles at least, continues during the steady state.

E. BSP-S^{35} distribution in CCl_4 treated dogs

As indicated in Figure 2, a steady state of blood and bile BSP concentrations was obtained more slowly in these animals than in the normals. BSP activity to color ratios in plasma and bile of these dogs did not differ significantly from those of normal dogs although the data of Table 3 suggest that a larger series might show that activity to color ratios in the bile of the CCl_4 treated dogs may be slightly lower than in normal dogs. Liver injury was reflected most clearly in low excretion rates and high plasma BSP concentrations considering the infusion rates employed (Table 3).

As a result of the high proportion of BSP-S^{35} retained in these dogs, the rest carcass concentrations of S^{35} were several times as high in those as in normal dogs; the ratio of rest carcass S^{35} to plasma S^{35} concentrations was found normal in the case of one and slightly subnormal in the case of the second animal. Renal S^{35} concentrations were high but no more so than would be predicted as a result of the high plasma or rest carcass concentrations. Hepatic BSP-S^{35} concentrations, relative to the carcass were normal, or at worst slightly below normal. As a result of the low biliary excretion, however, there was a significant reduction below normal in the proportion of infused dye which could be recovered from liver and bile.

DISCUSSION

Transfer of acid dyes from blood to bile was conceived of by early investigators as a relatively simple process, involving secretory activity of the hepatic (or possibly the Kupffer) cells to effect the manifold concentration of these dyes in the bile. More recently this picture has been complicated somewhat by data indicating a temporal separation between disappearance of the dye from the blood stream and its reappearance in the bile (5). Finally, dye balance studies during continuous BSP infusion (4) rendered the old picture altogether untenable: Only about 60 per cent of BSP infused can be recovered from liver and bile, and even during the so-called steady state a sizeable gap persists between infusion and excretion rates.

These studies, based on colorimetric assay, are supplemented by the present data involving BSP labeled with S^{35} in the sulfonate groups. Bile of anesthetized, acute bile fistula dogs treated with this material invariably contains more S^{35} than can be accounted for by colorimetric assay. These observations establish the fact that BSP undergoes some measure of metabolic transformation before being excreted.

That the fate of BSP does not include desulfonation (and hence, loss of its S^{35} label) emerges from several observations: In the present report it was shown that the excess S^{35} of bile does not occur as inorganic or as ethereal sulfate. In the blood stream, even after prolonged infusion no unaccounted S^{35} has been observed. These indications are strongly supported by more extensive investigations of the metabolism of BSP (to be reported elsewhere); all of the S^{35} activity administered to dogs as S^{35} BSP was recovered in the form of sulfonated phthaleins identical with or derived from BSP. These observations justify the use of S^{35} determinations as the basis of a study of the distribution of infused S^{35} BSP throughout the body.

The gap between the amount of BSP infused, and the amount recovered from liver and bile is narrowed considerably if the recovery of S^{35}, rather than that of colorimetric BSP is made the basis of consideration: Almost 80 per cent of the total dye removed from the circulation in normal dogs can now be accounted for in terms of hepatic extraction. This figure agrees well with an earlier estimate reached by different means (4) and with recent direct determinations of hepatic blood flow in comparison with estimated hepatic blood flow calculated from BSP data (11). The remainder of the infused dye is distributed throughout the tissues, as indicated by Tables 4 and 5.

Several aspects of the observed distribution of extrahepatic BSP are of theoretical interest: The high concentrations of BSP in tissues like kidney and upper gastrointestinal tract; the efficacy of the blood-brain barrier; the surprisingly high concentration of this virtually lipid insoluble dye in the fatty tissue; and the inferred low concentrations in the skeleton and skin. Particular interest attaches to the uptake of BSP by the skeletal muscles. From theoretical considerations (8) it would appear that the equilibrium concentration

in this tissue should approximate closely that of the blood, or even exceed it. This, indeed, is the situation encountered with respiratory muscle and with cardiac muscle. The large skeletal muscles, on the other hand, show concentrations of BSP considerably below those coexisting in the blood plasma; thus, this tissue apparently has not reached equilibrium with the blood even after several hours of BSP infusion. By direct experiment it could be shown that this conclusion is correct, and that throughout the period of infusion the concentration of BSP in at least one of the great muscles increases continuously, presumably towards an equilibrium value not too different from that in the respiratory muscles. Because of its great relative mass, this tissue accounts for a large proportion of the entire extrahepatic BSP store; the slowness with which the muscle mass is saturated with BSP leads one to predict that elimination of the uptake of BSP by this tissue cannot be achieved by an initial booster dose. BSP uptake by the large muscles thus remains at a significant level throughout the course of an infusion experiment, and constitutes the major extrahepatic sink for BSP.

Carbon tetrachloride poisoning does not greatly alter the distribution of retained BSP between liver and rest carcass. This would have been predicted from experiments with BSP uptake by liver slices *in vitro* (8), as well as from histological observations *in vivo* based on the related dye rose bengal (12). Apparently, the ability of the liver to store and to concentrate BSP and related dyes is unaffected by CCl_4 injury. The excretion of such dyes, on the other hand, is greatly decreased under these conditions. Since the total BSP extracted by the liver is the sum of BSP (or BSP derivatives) excreted into bile, and BSP stored in the liver, CCl_4 injury results in a markedly reduced hepatic BSP extraction under continuous infusion conditions, and in a greater proportion of extrahepatically retained dye. Only if the infusion rate could be slowed until excretion can keep pace with infusion (if, indeed, such a condition is possible in the presence of parenchymal injury) may a BSP distribution comparable to the normal be expected.

Finally, one may briefly consider the effect of the anesthesia and biliary tract surgery involved in the present experiments upon the results obtained. Both conditions have been claimed to affect BSP clearance after single injections; however, it was found in constant infusion experiments (4) that laparotomy (including preparation of a bile fistula as employed in the present series) did not result in blood BSP levels differing from those of dogs merely anesthetized, but receiving BSP at the same infusion rate.

In the isolated rat liver it has been shown (13) that Nembutal® does not directly interfere with either bile formation or BSP clearance. An indirect effect, mediated in the intact animal *via* changes in hepatic blood flow, is possible. Such an effect, however, would not alter the validity of the essential conclusions derived from the present experiments. Even if such an effect obtained, the result would be to render the conclusions derived from the study of "normal" dogs in the present series unduly conservative—the hepatic contribution to BSP extraction might actually be larger in the unanesthetized animal. In the presence of a functionally impaired liver, on the other hand, regardless of whether this is due to CCl_4 treatment alone, or compounded by anesthesia and surgery, the present data show that extrahepatic BSP uptake may become important.

CONCLUSIONS

The application of S^{35}-labeled sulfo-bromophthalein-sodium in the present report has provided definite evidence for the occurrence of chemical changes in the molecule of this dye, associated with the process of excretion into bile. Whether these reactions are an integral part of the mechanism of dye excretion by the liver or whether, like dye storage in the hepatic cells, they are only an incidental accompaniment of such excretion, remains to be established by future investigations. With regard to the distribution of infused BSP, the present results in anesthetized, acute bile fistula dogs confirm previous impressions regarding the otherwise normal dog. Hepatic BSP uptake accounts for a very large proportion of administered dye and errors in calculations based on the assumption that all of the BSP leaves the circulation on passage through the liver are relatively small. At the same time, findings of continued uptake of BSP by skeletal muscle point to this tissue as the major site of sustained extrahepatic dye extraction.

In dogs with toxic hepatitis the present findings allow a clearer formulation of ideas previously expressed concerning errors of quantitative interpretation caused by neglect of extrahepatic BSP uptake. It has been shown that the reduction in the hepatic BSP extraction is accounted for almost entirely in terms of decreased BSP excretion; if infusion conditions allowing BSP excretion to keep pace with BSP infusion could be attained in the face of parenchymal injury these should yield results comparable in reliability to those in the normal dog.

Finally, the present results clarify the nature of the so-called lost BSP observed previously in experiments confined to colorimetric assay alone.

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THYROXINE BINDING CAPACITY OF SERUM ALPHA GLOBULIN IN HYPOTHYROID, EUTHYROID, AND HYPERTHYROID SUBJECTS [1]

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Circulating thyroxine is associated with a specific serum alpha globulin (1–6). This thyroxine-binding globulin (TBG) appears to exist in plasma in quantity greater than is required to bind endogenous thyroxine, for it will bind additional thyroxine when added *in vitro* (6–8). This capacity of TBG to bind exogenous thyroxine *in vitro* appeared in preliminary studies (9) to depend upon the state of thyroid gland activity. The purpose of this paper is to report in more detail the capacity of the serum of hypothyroid, euthyroid, and hyperthyroid subjects to bind added thyroxine.

MATERIALS AND METHODS

All subjects studied were seen in the Department of Medicine of the University of Wisconsin. The subjects were either hypothyroid (nine), euthyroid (nine), or hyperthyroid (nineteen), as judged by symptoms, physical findings, 24-hour I^{131} uptake and other appropriate measurements. Two of the hypothyroid subjects were athyreotic following total thyroidectomy.

Ten micrograms of I^{131} labelled thyroxine [2] in propylene glycol were added to one milliliter of a solution containing 90 micrograms per milliliter of inert thyroxine. The latter solution was prepared from chromatographically purified thyroxine which had been quantitated gravimetrically. This procedure was adopted to minimize possible error in the quantitation of the labelled thyroxine. This mixture was then diluted to 10 ml. Appropriate aliquots of this solution were added to serum to yield a series of dilutions containing 0.01 to 1.0 micrograms per milliliter.

Fifty microliters of each of these mixtures were submitted to paper zone electrophoresis as previously described (3). On completion of the electrophoresis, the paper strips were dried and the radioactivity determined by use of a chromatogram scanner. The distribution of radiothyroxine was obtained by computing with a planimeter the area under the curves produced by the recording galvanometer of the scanner.[3] The amount of thyroxine bound by the TBG was estimated as follows:

$$\text{TBG-bound thyroxine} = \frac{\text{Area TBG}}{\text{Total area}} \times \text{thyroxine added}$$

RESULTS

In all sera examined the thyroxine appeared in the alpha globulin zone. At the lowest concentrations used (0.01 to 0.05 micrograms per milliliter) only a very small amount of thyroxine appeared in the albumin. As the concentration of thyroxine was increased from 0.1 to 1.0 micrograms per milliliter, a greater proportion of thyroxine appeared in the albumin as the alpha globulin binding capacity appeared to become relatively saturated. In Figure 1 are shown representative curves of distribution of radiothyroxine following electrophoresis of sera from euthyroid, hypothyroid, and hyperthyroid subjects. It is evident that there is a greater accumulation of radiothyroxine in the TBG of the hypothyroid subject and conversely less in the hyperthyroid.

Figure 2 is a scattergram showing the relation between the quantity of radiothyroxine added to serum and that bound by the TBG. In the lower concentrations (0.05 to 0.4 micrograms per milliliter) this relationship is roughly linear. At higher concentrations (0.4 to 1.0 micrograms per milliliter) relatively less of the added radiothyroxine appears in the TBG. A plateau is thereby produced, which is believed to reflect a "saturation" of the TBG. The concentration at which "saturation" occurs varies with the state of thyroid activity; being approximately 0.15 to 0.2 micrograms radiothyroxine per milliliter in the hy-

[1] This work was supported in part by the Wisconsin Alumni Research Foundation.

[2] Obtained from Abbott Laboratories, Oak Ridge, Tennessee. (Preparations not at least 95 per cent pure were repurified by paper chromatography.)

[3] By adding varying quantities of radiothyroxine in spots along a paper strip it was possible to demonstrate a linear relationship between the amount of radioactivity and the area under the curve regardless of the geometry of the spot.

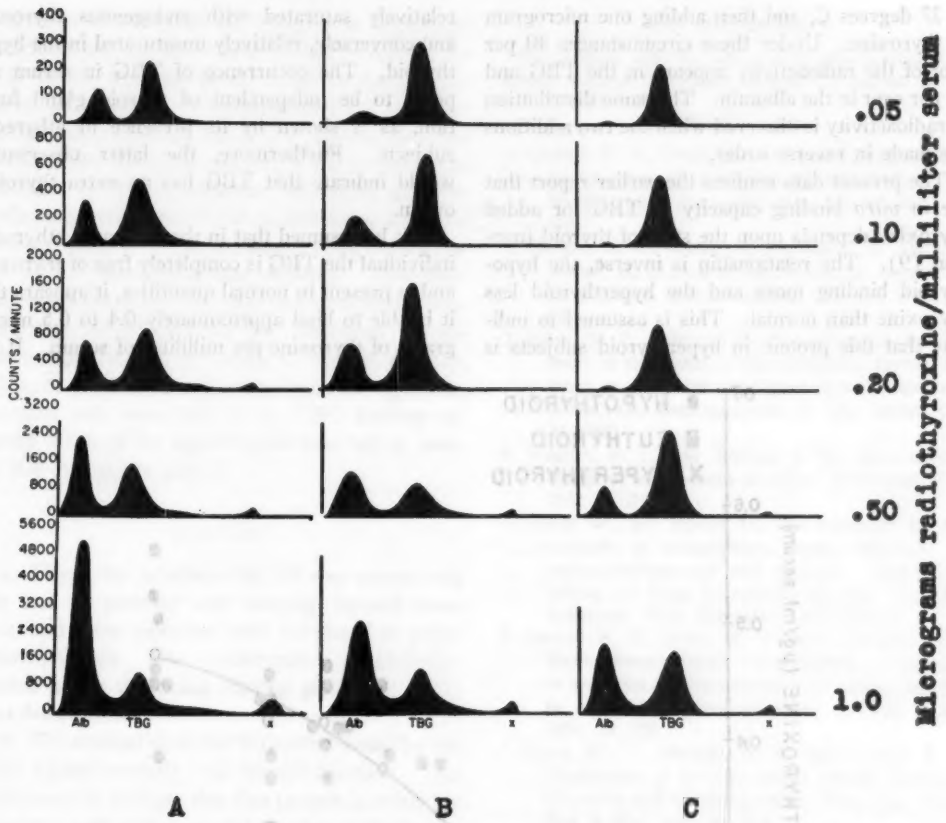

FIG. 1. DISTRIBUTION OF RADIOTHYROXINE FOLLOWING ELECTROPHORESIS OF SERA FROM (A) HYPERTHYROID, (B) EUTHYROID, AND (C) HYPOTHYROID

x—indicates starting point of electrophoresis
TBG—indicates thyroxine binding globulin
Alb—indicates albumin

As the radioactivity of the preparations increased, narrower slit-openings were used in the scanner, hence there is no absolute relationship of the radioactivity from one concentration to the next.

perthyroid, 0.25 micrograms per milliliter in the euthyroid, and 0.4 to 0.5 micrograms per milliliter in the hypothyroid.

DISCUSSION

The capacity of TBG to bind thyroxine is relatively specific. Triiodothyronine also associates with this protein but it can be displaced readily with thyroxine while the converse is not so (7). The presence of large quantities (10 times molar concentration of thyroxine) of tyrosine, diiodotyrosine, diiodothyronine and iodide ion fails to interfere with the TBG thyroxine binding (10). This specific binding in the normal is observed only when the quantity of added thyroxine is less than 0.1 micrograms per milliliter. When the concentration exceeds this level, the thyroxine can be found in all protein fractions with albumin the main secondary carrier.

The radiothyroxine added *in vitro* appears to exchange freely with the thyroxine previously bound. This can be demonstrated by adding 0.1 microgram radiothyroxine to one milliliter of normal serum, allowing 20 minutes of incubation

at 37 degrees C, and then adding one microgram of thyroxine. Under these circumstances 40 per cent of the radioactivity appears in the TBG and 60 per cent in the albumin. The same distribution of radioactivity is observed when the two additions are made in reverse order.

The present data confirm the earlier report that the *in vitro* binding capacity of TBG for added thyroxine depends upon the state of thyroid function (9). The relationship is inverse, the hypothyroid binding more and the hyperthyroid less thyroxine than normal. This is assumed to indicate that this protein in hyperthyroid subjects is relatively saturated with endogenous thyroxine and conversely, relatively unsaturated in the hypothyroid. The occurrence of TBG in serum appears to be independent of thyroid gland function, as is shown by its presence in athyreotic subjects. Furthermore, the latter observation would indicate that TBG has an extra thyroidal origin.

If it be assumed that in the untreated athyreotic individual the TBG is completely free of thyroxine and is present in normal quantities, it appears that it is able to bind approximately 0.4 to 0.5 micrograms of thyroxine per milliliter of serum. If the

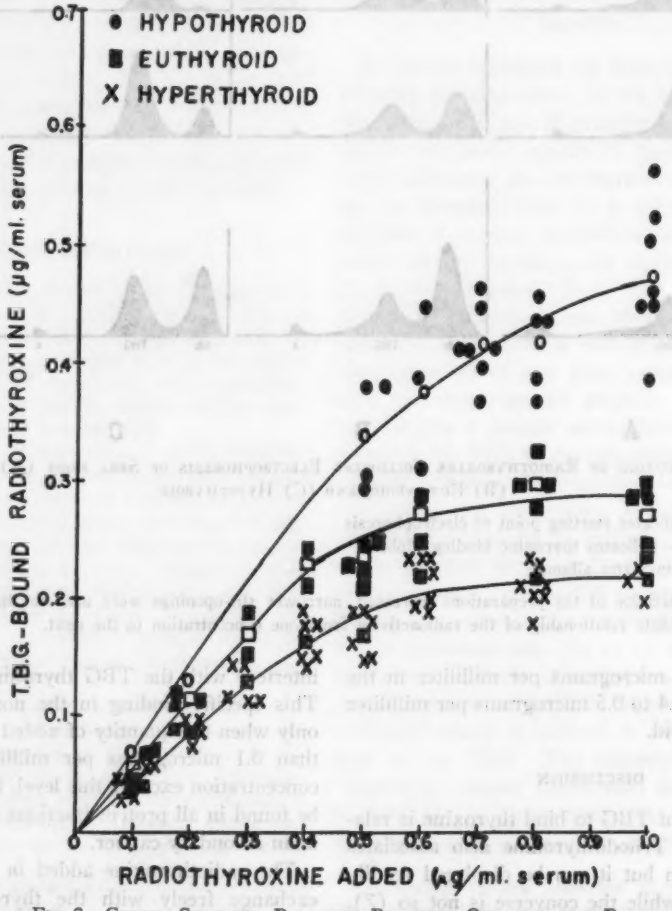

FIG. 2. GRAPH SHOWING RELATION BETWEEN QUANTITY OF RADIOTHYROXINE ADDED TO SERUM AND THAT BOUND BY THE THYROXINE-BINDING-GLOBULIN (TBG)
Open symbols indicate the mean.

plasma concentrations of endogenous thyroxine calculated from PBI determinations are taken as 0.00 to 0.05 micrograms per milliliter for the hypothyroid, 0.10 to 0.15 micrograms per milliliter for the euthyroid, and 0.2 to 0.25 micrograms per milliliter for the hyperthyroid, it can be seen that the sum of this endogenous thyroxine and the added exogenous thyroxine as estimated from the present data, is in each category approximately 0.4 to 0.5 micrograms per milliliter. This suggests that there may be little variation in the concentration of TBG from one individual to another regardless of the state of thyroid activity. Thus, in the normal subject endogenous thyroxine occupies only one-third of the TBG binding capacity, while in the hyperthyroid one-half or more of this capacity is utilized.

SUMMARY

1. Thyroxine labelled with I^{131} was mixed with the sera of patients with varying thyroid function, and these mixtures were subjected to paper electrophoresis. The concentration of radiothyroxine in the thyroxine binding globulin (TBG) was determined.

2. The amount of added thyroxine bound by the TBG varies inversely with thyroid function. This is assumed to indicate that this protein is relatively saturated with endogenous thyroxine in the hyperthyroid and relatively unsaturated in the hypothyroid.

3. From evidence in an athyreotic individual it appears that the total thyroxine binding capacity of the TBG is 0.4 micrograms per milliliter, only one-third of which is utilized in the normal subject.

ACKNOWLEDGMENT

The authors wish to acknowledge the invaluable assistance of Miss Sue Ames, Medical Technician.

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ISOTOPIC STUDIES OF PLASMA CHOLESTEROL OF ENDOGENOUS AND EXOGENOUS ORIGINS[1]

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This study was undertaken in order to compare the metabolic behavior of plasma cholesterol derived from the diet with that of cholesterol synthesized *in vivo* from acetate (1). Cholesterol labeled with either isotopic carbon or hydrogen was administered orally to human subjects and the incorporation into plasma cholesterol was followed over an extended period. In certain instances, both endogenous and exogenous cholesterol metabolism were studied simultaneously by the use of appropriately labeled acetate and cholesterol. These techniques were also applied to an examination of the behavior of plasma cholesterol in four patients with hypercholesterolemia.

EXPERIMENTAL

Subjects. The patients were hospitalized in the James Ewing Hospital unit of Memorial Center; all were in good clinical condition and a summary of pertinent data appears in Table I. Of the seven patients reported, three had normal plasma cholesterol levels and four had hypercholesterolemia of varied etiology.

Isotopic materials. The precise dosages of the labeled compounds are recorded in Table I. Cholesterol, either radiohydrogen (tritium or H^3), heavy hydrogen (deuterium or H^2), or radiocarbon (C^{14}) labeled, was dissolved in sesame oil and fed in gelatin capsules. Sodium acetate labeled either with C^{14} or H^3 was dissolved in 100 ml. of tap water and given orally. The quantity of radiation delivered by C^{14} to the patients as well as precautions to be observed in using this isotope in humans have already been described (1). Approximately 6 millicuries (mc.) of H^3 are required to deliver the tolerance dose of radiation or 0.3 rep. per week, if this isotope were completely retained and equally distributed through the body. As the amount of H^3 administered was considerably below this level, and since a large fraction of the dose was not retained, the quantity of radiation the patient receives from the isotope is far below tolerance levels.

The tritium-labeled sodium acetate with a specific activity of 0.55 mc. per millimole was made in this laboratory by exchange of malonic acid with tritium enriched water by the method of Halford and Anderson for preparation of deuterium-labeled acetate (2). Acetate-2-C^{14} was obtained from a commercial source and had a specific activity of 1 mc. per millimole. Cholesterol-4-C^{14} was prepared in this laboratory from cholestenone-4-C^{14} by the method of Belleau and Gallagher (3). The cholestenone-4-C^{14} was obtained from the Oak Ridge National Laboratory, and had a specific activity of 2.3 mc. per millimole. Deuterium labeled cholesterol was prepared by catalytic exchange of cholesterol with heavy water in the manner described by Bloch and Rittenberg (4). Tritium-labeled cholesterol, with the isotope chiefly at positions C-14 and C-15, was made in this laboratory by the following procedure which has not been described previously.

A solution of one gram of Δ^{14}-cholestene-3β-ol acetate (5), 5 ml. (10 curies) of tritium acetic acid, and 30 ml. of anhydrous ether was reduced with 254 mg. of Adams' catalyst and hydrogen gas until the absorption ceased. The crude cholestanyl acetate was treated with a dilute solution of perbenzoic acid in benzene overnight at room temperature. Ten grams of non-isotopic cholestanyl acetate were added to the mixture and the diluted cholestane-3β-ol-14,15-*t* acetate was purified by chromatography and recrystallization. Saponification of the acetate followed by oxidation of cholestanol with sodium dichromate gave eight grams of cholestane-3-one-14,15-*t* which was then converted to four grams of Δ^4-cholestene-3-one (6). Sodium borohydride reduction (3) of the enol acetate prepared from the unsaturated ketone afforded cholesterol-14,15-*t*, m.p. 149 to 149.5°.

Blood samples of thirty to forty ml. were collected at frequent intervals in either oxalated or heparinized tubes for the isolation of free and ester cholesterol as the digitonide by the procedure described elsewhere (7). In those cases where cholesterol-4-C^{14} was administered, collections of expired carbon dioxide were made in order to determine whether the steroid nucleus was broken

[1] This investigation was carried out under contract AT(30-1)-910 with the United States Atomic Energy Commission and supported in part by research grants (C-440) from the National Cancer Institute and (H-564) from the National Heart Institute, of the National Institutes of Health, United States Public Health Service.

TABLE I

Clinical data on patients

Patient	Sex	Age	Wgt. (lbs.)	Diagnosis	Plasma cholesterol mg./100 ml. Total	Free	Labeled materials Compound	Weight	Activity
CHC-1	M	58	130	Pancreas carcinoma	150	50	Cholesterol-4-C^{14} Acetate-2-H^3	15.4 mg. 98 mg.	91 μc. 700 μc.
CHC-2	F	30	115	Functioning adrenal cortical carcinoma	235	62	Cholesterol-4-C^{14} Acetate-2-H^3	10.1 mg. 67 mg.	60 μc. 480 μc.
CHC-3	F	34	131	Cushing's syndrome	200	66	Cholesterol-4-C^{14} Acetate-2-H^3	9.5 mg. 67 mg.	56 μc. 480 μc.
CHC-4	F	56	115	Hypercholesterolemia Xanthoma tendinosum	410	106	Cholesterol-4-C^{14}	20.6 mg.	122 μc.
CHC-5	F	41	116	Hypercholesterolemia Xanthoma tendinosum	408	122	Cholesterol-4-C^{14} Cholesterol-H^3	10.7 mg. 1.0 g.	63 μc. 11.87 AD*
CHC-6	M	43	160	Nephrotic syndrome	420	100	Cholesterol-4-C^{14}	10.7 mg.	63 μc.
CHT-1	F	49	115	Hypercholesterolemia Xanthoma tuberosum	812	301	Acetate-2-C^{14} Cholesterol-H^3	16 mg. 7.5 mg.	200 μc. 113 μc.

* AD = atom per cent excess deuterium.

down to compounds that might be oxidized to carbon dioxide. Urine and feces were obtained for variable periods of time to measure the excretion of the administered radioactivity through these routes, as well as for the isolation of steroid metabolites. These latter data will be presented in detail in a separate communication, although preliminary results have been reported (8, 9).

Radioactivity measurements. The methods used for the measurement of radioactivity of cholesterol digitonide samples containing only C^{14} have been previously reported (1). The tritium analyses were carried out on cholesterol digitonide by proportional counting in the gas phase by the method described by Eidinoff (10).

In those experiments in which acetate and cholesterol were given simultaneously, the cholesterol digitonide isolated from the plasma contained both isotopes. The digitonide was burned in a combustion apparatus similar to that described by Glascock (11) and simultaneous collection of both water and carbon dioxide was made. The water was converted to hydrogen and counted in the gas phase in the proportional region (10). Radiocarbon activity was assayed as carbon dioxide admixed with carbon disulfide in the Geiger-Müller region (12).

The carbon-14 content of certain solid samples containing both carbon-14 and tritium was determined in windowless flow gas counters with an aluminum foil (0.13 mg. per cm^2) placed over the planchet to absorb the tritium beta particles. This foil removed approximately 90 per cent of the tritium radiation, but permitted about 80 per cent of the carbon beta particles to pass. An accurate estimation of the tritium content of a sample could not be made by this procedure, but since a constant portion of the carbon radiation passed through the foil, it was adequate for the measurement of the carbon-14 content of a sample containing both isotopes.[2]

$$C = C_0 \frac{C_s/C_0 - f_{tr}}{f_e - f_{tr}}$$

C = true carbon count

C_0 = net count unshielded

C_s = net count shielded

f_e = fraction of C^{14} transmitted through shield

f_{tr} = fraction of H^3 transmitted through shield

f_e and f_{tr} were determined once each week for each aluminum shield. The f_e was measured by counting a shielded sample containing C^{14} only, while f_{tr} was determined in the same manner with a sample containing only H^3.

The C^{14} content of urine was measured with 0.2 ml. of urine dried on a planchet of 4.9 cm^2 area containing a piece of lens paper of the same dimensions. The lens

[2] Calculation for determining radiocarbon by solid counting in the presence of tritium:

TABLE II

Radioactivity of plasma cholesterol in subjects with normal cholesterol levels

CHC-1					CHC-2					CHC-3		
From cholesterol-4-C^{14}			From acetate-2-H^3		From cholesterol-4-C^{14}			From acetate-2-H^3		From cholesterol-4-C^{14}		
Time*	Free†	Ester†	Free‡	Ester‡	Time*	Free†	Ester†	Free‡	Ester‡	Time*	Free†	Ester†
0.125	22.0	3.18	26.9	2.95	0.167	118	93.5	27.8	5.32	0.184	134	117.5
0.35	61.0	43.0	16.4	6.67	0.39	722	610	18.7	5.37	0.417	446	422
0.73	81.2	85.4	12.5	7.04	0.94	1,500	1,030	8.71	7.38	0.805	681	414
1.2	135.5	137.5	11.8	6.75	1.27	1,620	1,110	8.69	7.72	1.11	622	562
1.75	125	151.5	9.16	8.44	2	1,420	1,280	10.92	9.65	1.84	584	601
2.2	144	174.5	9.21	7.84	3	1,180	1,280	8.86	9.23	2.18	554	664
2.75	137	179.5	9.21	5.57	4	941	1,240	6.49	8.44	2.83	476	612
3.2	129.5	167	7.80	7.65	5	840	1,120	6.17	8.15	4.0	452	575
3.75	129.0	173	6.48	8.05	7	643	865		6.55	6	344	488
					10	535	711	4.98	3.96	7	286	423
										8	217	368
										11	148	240
										13	139	198
										14	134	188

* Elapsed time in days after administration of labeled material.
† $\times 10^3$ DPM per mM cholesterol.
‡ $\times 10^3$ DPM per mM H_2.

TABLE III

Radioactivity of plasma cholesterol after administration of cholesterol-4-C^{14} to hypercholesterolemic subjects

CHC-4			CHC-5			CHC-6		
Time*	Free†	Ester†	Time*	Free†	Ester†	Time*	Free†	Ester†
0.04	98.5	—	0.167	24.3	11.5	0.33	320	408
0.62	1,112	—	0.375	126.7	64.3	1	338	622
1.1	1,760	—	0.845	215	124.7	2	417	604
1.6	1,750	—	1.06	246	204	3	425	437
2	1,575	—	1.44	255	204	5	357	404
2.6	1,480	—	1.44	0.060§	0.045§	7	297	338
3.1	1,420	—	ratio‡	0.24‡	0.22‡	10	239	266
3.6	1,335	—	1.87	275	214	14	179	215
5.6	974	—	2.87	210	255	17	154	169
6.6	982	—	2.87	0.048§	0.057§	22	104	117.5
7.6	830	1,084	ratio‡	0.23‡	0.22‡	27	101	93.7
8.6	741	—	3.9	210	256	34	69.0	77.3
9.6	728	988	5	167	207	41	59.4	68.7
10.6	660	864	6	162	197	48	56.6	74.0
11.6	587	791	7	121.5	185	55	43.3	55.7
12.6	552	718	8	116	172	62	33.8	44.8
14.6	556	718	10	111	152	69	32.3	36.4
16.6	441	597	13	93.2	111	76	33.6	37.2
20.6	413	484	13	0.022§	0.026§	83	39.2	—
21.6	396	457	ratio‡	0.24‡	0.23‡	97	24.0	25.4
23.6	363	397	15	71.5	106	110	20.7	23.9
26.6	324	381	17	62.8	94.4	124	17.5	—
29.6	302	315	20	58.6	82.6	138	12.5	—
33.6	236	326	22	57.6	76.4			
36.6	197	308	24	60.3	72.2			
140.0	59.0	82.3	28	46.7	62.0			
			35	49.7	54.0			
			43	42.3	46.3			
			52	39.0	38.9			
			66	27.8	33.2			
			76	27.6	31.2			
			92	19.7	20.5			
			105	—	—			
			144	17.0	14.3			
			167	12.8	13.9			

* Elapsed time in days after administration of labeled material.
† $\times 10^3$ DPM per mM cholesterol.
‡ Ratio of deuterium content $\times 10^3$ to radiocarbon content.
§ Atom per cent excess deuterium in cholesterol (AD).

paper facilitated even distribution over the surface of the planchet to give plates of uniform thickness. Feces were extracted three times in a Waring Blendor with three volumes of acetone. A portion of the acetone extract was plated on lens paper in the manner described for urine.

The various counting procedures were intercalibrated with a reference sodium carbonate-C^{14} standard obtained from the National Bureau of Standards. The radioactivity of cholesterol samples containing carbon-14 is expressed in units of "disintegrations per minute per millimole" (DPM per mM). The tritium radioactivity data are expressed in units of "disintegrations per minute per millimole of hydrogen" (DPM per mM H_2).

RESULTS

Three patients, CHC-1, CHC-2, CHC-3, with normal plasma cholesterol levels each received from 50 to 90 microcuries (μc.) of cholesterol-4-C^{14} and from 500 to 700 μc. of tritium-labeled acetate. Three other patients, CHC-4, CHC-5, CHC-6, with elevated plasma cholesterol levels, received from 60 to 120 μc. of cholesterol-4-C^{14}; 1.016 gm. of cholesterol containing 11.87 atoms per cent excess deuterium was given to subject CHC-5 along with the C^{14} cholesterol. An additional patient with hypercholesterolemia, CHT-1, received 113.2 μc. of tritium labeled cholesterol and 200 μc. of acetate-2-C^{14}.

The specific activities of free and ester cholesterol isolated from patients CHC-1, CHC-2, CHC-3 are listed in Table II. Table III contains the specific activity data from subjects CHC-4, CHC-5, CHC-6, while the results obtained in CHT-1 are listed in Table IV.

The data obtained for four and a half days following the simultaneous administration of acetate-H^3 and cholesterol-4-C^{14} to subject CHC-1 are plotted in Figure 1. As shown on the lower right portion of this figure, the specific activity of free cholesterol synthesized *in vivo* from acetate is at a maximum with the earliest sample and then declines rather rapidly. The ester cholesterol derived from acetate is at a minimum value with the first sample and then gradually rises until it intersects the free curve at approximately three days, after which the specific activity of the ester cholesterol exceeds that of the free. For comparison with these tritium data, specific activity curves previously reported from a patient who received

TABLE IV

Radioactivity of plasma cholesterol in a hypercholesterolemic subject given acetate-2-C^{14} and cholesterol-H^3

CHT-1

	From acetate-2-C^{14}		From cholesterol-H^3	
Time*	Free†	Ester†	Free‡	Ester‡
0.125	568	18.1	3.3	2.79
0.33	535	53.7	25.3	27.3
0.80	450	85.7	41.7	43.0
1.26	372	122.8	41.1	67.8
1.73	365	126.5	50.3	68.8
2.73	263	154.2	49.8	78.6
3.73	222	155.0	48.6	66.0
4.73	180	172.0	42.8	63.6
6.73	166	151.8	41.5	54.2
8.73	154	158.2	36.4	46.2
10.73	118	129.5	29.2	35.3
13.73	108	117.5	28.2	31.9
17.73	88.4	102.0	25.1	28.6
22	82.4	86.0	21.0	25.8
25	61.4	85.5	18.6	22.4
29	—	—	—	—
32	53.6	68.5	15.8	18.1
36	52.8	62.6	14.8	16.2
39	50.0	55.0	13.7	15.8
43	44.8	55.7	12.7	15.6
49	43.3	48.4	11.95	13.9
61	36.6	37.0	—	11.2
86	28.9	33.8	8.15	11.85
115	20.4	23.3	6.62	6.12

* Elapsed time in days after administration of labeled material.

† $\times 10^3$ DPM per mM cholesterol.

‡ $\times 10^3$ DPM per mM H_2.

C^{14}-labeled acetate (1) are shown in the upper right portion of Figure 1. The behavior of cholesterol synthesized from acetate is similar whether the acetate is labeled with either tritium or C^{14}, and these data and others to be presented demonstrate the interchangeable use of these two isotopes for the study of the conversion of acetate to cholesterol.

The activity of plasma free cholesterol originating in the diet is at a minimum value with the 3-hour sample, as illustrated on the left portion of Figure 1. The specific activity then rises to a peak at about 1.5 to 2 days and afterwards declines. The radioactivity of the "exogenous" plasma ester cholesterol is also at a minimum with the first sample, rises less rapidly than that of the free sterol, and reaches a peak value at about 2.5 days, having intersected the free curve at about 1 day. After this crossover point, the ester specific activity is greater than that of the free for the duration of the period of observation.

Figure 2 compares the incorporation of dietary

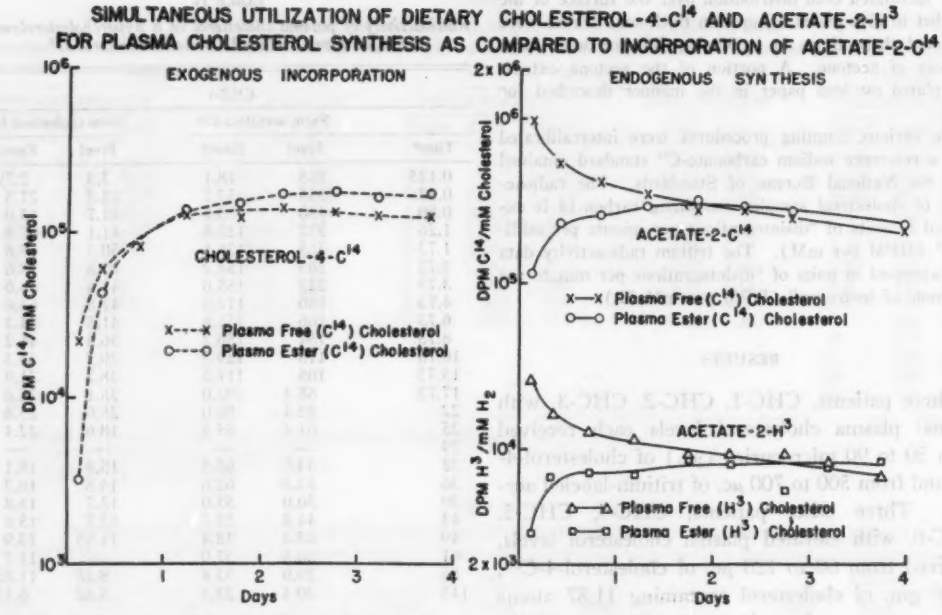

FIG. 1. THE SIMULTANEOUS UTILIZATION OF INGESTED CHOLESTEROL-4-C^{14} AND ACETATE-2-t FOR PLASMA CHOLESTEROL SYNTHESIS AS COMPARED WITH THE INCORPORATION OF ACETATE-2-C^{14}

cholesterol-4-C^{14} into plasma cholesterol of a subject with a normal cholesterol level (CHC-2) with a patient who had hypercholesterolemia accompanying the xanthoma tendinosum syndrome (CHC-5). It can be seen that the mode of rise of free and ester cholesterol, the points of intersection, and the subsequent rate of decline over a ten-day period, are virtually indistinguishable. The specific activity of the plasma cholesterol in the normocholesterolemic patient is higher; however, this difference may be related to individual variation in the quantity of labeled cholesterol that had been absorbed. The dilution of the administered labeled sterol by the larger plasma cholesterol pool in the hypercholesterolemic might also explain the lower specific activity.

In Figures 3 and 4, results obtained over a longer period are presented for comparison of the rates of decline of endogenously and exogenously derived cholesterol. The plasma free cholesterol specific activities measured after the administration of labeled cholesterol to the hypercholesterolemic subject, CHC-4, are plotted in Figure 3 together with the specific activities of plasma free cholesterol of a normocholesterolemic subject who received labeled acetate. The acetate data have been previously reported (1). With the exception of the early differences in the behavior of endogenously or exogenously derived plasma cholesterol already described above, it will be noted that the radioactivity of free cholesterol whether absorbed from the diet or made *in vivo*, declines at a similar rate in these two patients during the 30-day period of observation. In Figure 4, the equivalent data for ester cholesterol in the hypercholesterolemic subject CHC-5 and the typical acetate subject are shown and the similar rates of decline of sterol radioactivity in these dissimilar patients are again apparent.

Subject CHT-1 who had hypercholesterolemia associated with the xanthoma tuberosum syndrome received cholesterol-H^3 and acetate-2-C^{14} simultaneously. This patient was studied for six months and Figure 5 is a comparison of the behavior of cholesterol arising from these two sources for the first 43 days. The findings in this patient differ in two respects from those obtained in normocholesterolemic subjects and in

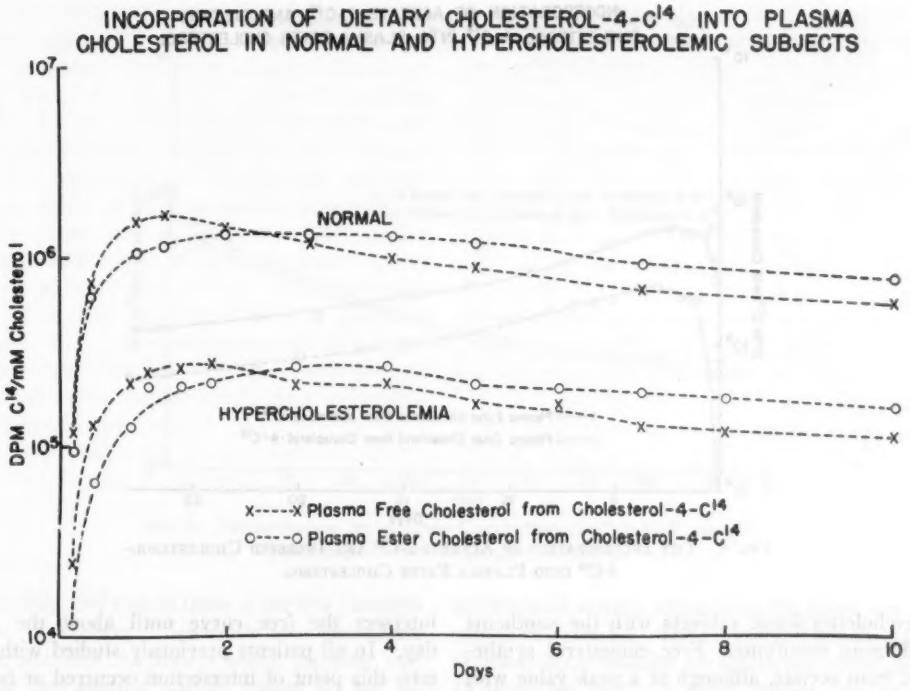
INCORPORATION OF DIETARY CHOLESTEROL-4-C14 INTO PLASMA CHOLESTEROL IN NORMAL AND HYPERCHOLESTEROLEMIC SUBJECTS
DPM C14/mM Cholesterol
NORMAL
HYPERCHOLESTEROLEMIA
x----x Plasma Free Cholesterol from Cholesterol-4-C14
o----o Plasma Ester Cholesterol from Cholesterol-4-C14
Days

FIG. 2. THE INCORPORATION OF INGESTED CHOLESTEROL-4-C^{14} INTO PLASMA CHOLESTEROL IN SUBJECTS WITH NORMAL CHOLESTEROL LEVELS AND IN HYPERCHOLESTEROLEMIA

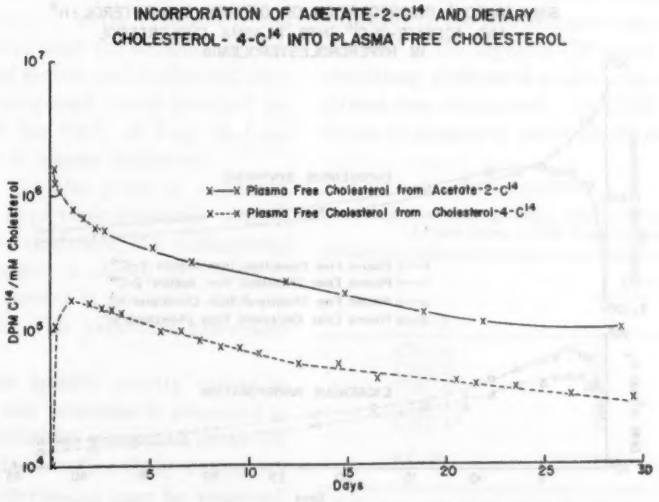
INCORPORATION OF ACETATE-2-C14 AND DIETARY CHOLESTEROL- 4-C14 INTO PLASMA FREE CHOLESTEROL
DPM C14/mM Cholesterol
x——x Plasma Free Cholesterol from Acetate-2-C14
x----x Plasma Free Cholesterol from Cholesterol-4-C14
Days

FIG. 3. THE INCORPORATION OF ACETATE-2-C^{14} AND INGESTED CHOLESTEROL-4-C^{14} INTO PLASMA FREE CHOLESTEROL

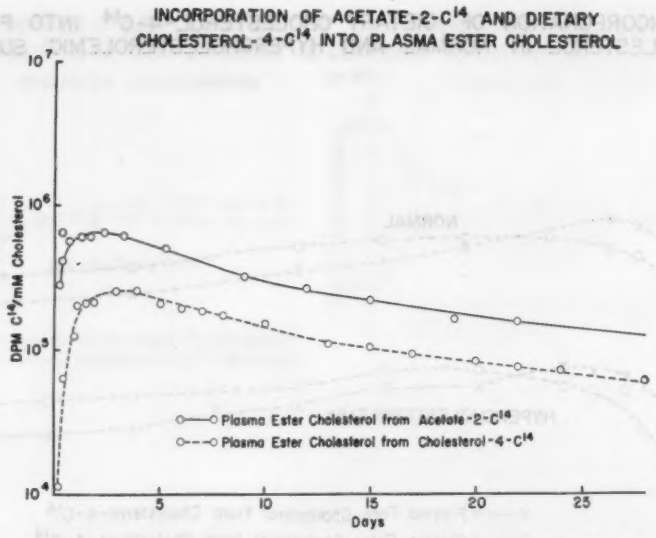
INCORPORATION OF ACETATE-2-C14 AND DIETARY CHOLESTEROL-4-C14 INTO PLASMA ESTER CHOLESTEROL
DPM C14/mM Cholesterol
Plasma Ester Cholesterol from Acetate-2-C14
Plasma Ester Cholesterol from Cholesterol-4-C14
Days

FIG. 4. THE INCORPORATION OF ACETATE-2-C^{14} AND INGESTED CHOLESTEROL-4-C^{14} INTO PLASMA ESTER CHOLESTEROL

hypercholesterolemic subjects with the xanthoma tendinosum syndrome. Free cholesterol synthesized from acetate, although at a peak value with the initial sample, declines more slowly during the first few days of observation, so that the rising specific activity curve of ester cholesterol does not intersect the free curve until about the sixth day. In all patients previously studied with acetate, this point of intersection occurred at two to three days (1). Plasma cholesterol derived from the diet, differs in that the ester cholesterol specific activities are initially at a higher level, rise

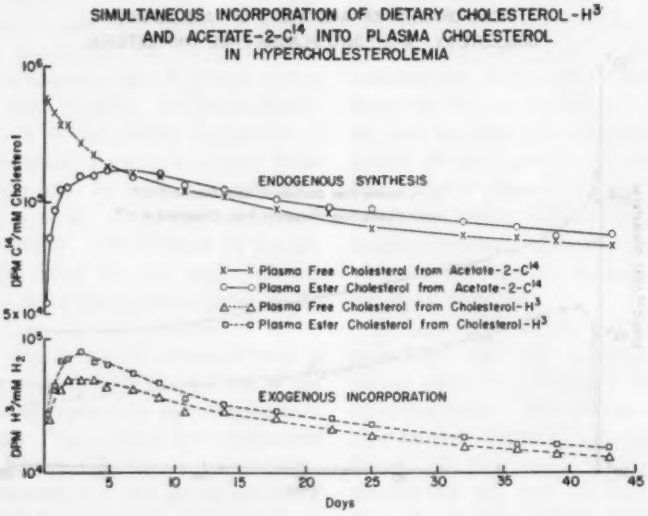
SIMULTANEOUS INCORPORATION OF DIETARY CHOLESTEROL-H3 AND ACETATE-2-C14 INTO PLASMA CHOLESTEROL IN HYPERCHOLESTEROLEMIA
DPM C14/mM Cholesterol
ENDOGENOUS SYNTHESIS
Plasma Free Cholesterol from Acetate-2-C14
Plasma Ester Cholesterol from Acetate-2-C14
Plasma Free Cholesterol from Cholesterol-H3
Plasma Ester Cholesterol from Cholesterol-H3
DPM H3/mM H2
EXOGENOUS INCORPORATION
Days

FIG. 5. SIMULTANEOUS INCORPORATION OF INGESTED CHOLESTEROL-*i* AND ACETATE-2-C^{14} INTO PLASMA CHOLESTEROL IN HYPERCHOLESTEROLEMIA

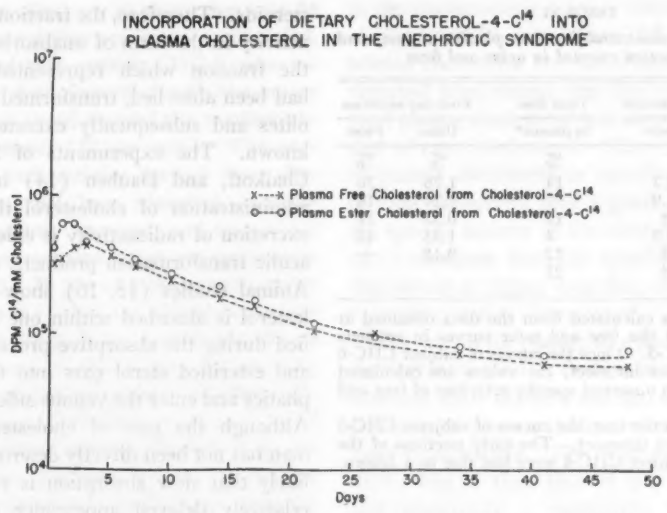

FIG. 6. INCORPORATION OF INGESTED CHOLESTEROL-4-C^{14} INTO PLASMA CHOLESTEROL IN THE NEPHROTIC SYNDROME

more rapidly, and exceed those of the free throughout the experiment. Since the free cholesterol specific activities are always below the ester values, the two specific activity curves do not intersect. The latter parts of the curves in Figure 5 show parallel rates of decline of cholesterol radioactivity similar to those observed in other patients with hypercholesterolemia and in patients with normal plasma cholesterol levels (Figures 3 and 4). These data derived after the simultaneous administration of labeled acetate and cholesterol show that either labeled compound yields identical results when used for the study of long term aspects of the behavior of plasma cholesterol.

Cholesterol-4-C^{14} was also given to a patient, CHC-6, who had hypercholesterolemia accompanying the nephrotic syndrome; the radioactivity of the plasma cholesterol is shown in Figure 6. Its behavior in this patient is similar to that observed in the subject with xanthoma tuberosum CHT-1.

An analysis of the specific activity curves in terms of component rate processes is presented in Table V and the similarities mentioned above for the decline of cholesterol radioactivity during the latter part of the experiments may be observed.

The radioactivity excreted in urine and feces in patients who received C^{14} cholesterol, as well as the levels of activity obtained in the blood, are recorded in Table VI. The recovery of administered radioactivity in feces by the fourth day varied from 14 to 48 per cent. A much smaller proportion, from 0.35 to 1.76 per cent, of the administered material was excreted in the urine during the same time interval. In the subjects whose plasma cholesterol specific activity curves exhibited a crossover of the free and ester cholesterol, 4 to 14 per cent of the ingested C^{14} sterol was present in circulating cholesterol at the peak radioactivity of plasma free cholesterol. In CHT-1 and CHC-6, where no crossover occurred, 27 and 12 per cent,

TABLE V

Component rates of decay curve of plasma cholesterol derived from labeled dietary cholesterol

Subject	Decay rates a $t_{\frac{1}{2}}$	Decay rates b $t_{\frac{1}{2}}$
CHC-4	22	3.0
CHC-5	22	2.5
CHC-6	20	4.0
CHT-1	19	1.9
CHT-1*	17	1.4
AC-3†	23.0	2.3

* Components of curves derived from acetate-2-C^{14} given simultaneously with cholesterol-H^3 to subject CHT-1.

† Components of curve derived from acetate-2-C^{14} as previously reported (1).

TABLE VI

Amount of labeled cholesterol found in plasma as compared with the fraction excreted in urine and feces

Subject	Intersection	Total dose	Four-day excretion	
	Days	In plasma*	Urine	Feces
		%	%	%
CHC-2	2.2	14	1.76	26
CHC-3	1.9	7	1.24	14
CHC-4	†	12	0.35	27
CHC-5	2.9	4	1.35	48
CHC-6	†	12	0.18	—
CHT-1	†	27		

* This value was calculated from the data obtained at the intersection of the free and ester curves in subjects CHC-2, -3, -4, and -5. Since the curves of subject CHC-6 and CHT-1 did not intersect, the values are calculated from the maximum observed specific activities of free and ester cholesterol.

† As discussed in the text, the curves of subjects CHC-6 and CHT-1 did not intersect. The early portions of the "ester" data in subject CHC-4 were lost due to a laboratory accident.

respectively, of the labeled dietary cholesterol was found in the plasma cholesterol at the maximum specific activity of the free fraction.

Expired carbon dioxide had no measureable radioactivity; as little as 0.1 per cent of the administered dose per day could have been detected by the method used.

DISCUSSION

Absorption and excretion of labeled cholesterol

The specific activity of plasma cholesterol would rise rapidly to an early peak, if the process of cholesterol absorption from the intestine was relatively rapid. This would be analogous to the behavior of plasma glucose after a glucose tolerance test. However, the experimental results in man show that the peak specific activity is not reached until approximately the second or third day. This suggests either that absorption is slow, that absorbed cholesterol is retained in the intestinal mucosa, or that newly absorbed cholesterol is rapidly removed from the blood and then later reintroduced into the circulation. Biggs and his co-workers (13) concluded that cholesterol absorption was slow, since they observed a sustained fecal excretion of radioactivity after feeding tritium labeled cholesterol. However, these authors measured either the total fecal radioactivity, or in one instance, the activity of a crude extract that contained cholesterol, coprosterol and possibly other steroids. Therefore, the fraction of the total radioactivity in the form of unabsorbed cholesterol and the fraction which represented cholesterol that had been absorbed, transformed into other metabolites and subsequently excreted in feces is unknown. The experiments of Siperstein, Jayko, Chaikoff, and Dauben (14) indicate that after administration of cholesterol the sustained fecal excretion of radioactivity is chiefly in the form of acidic transformation products, such as bile acids. Animal studies (15, 16) show that dietary cholesterol is absorbed within one day and is esterified during the absorptive process. Both the free and esterified sterol pass into the intestinal lymphatics and enter the venous side of the circulation. Although the rate of cholesterol absorption in man has not been directly determined, it seems unlikely that slow absorption is responsible for the relatively delayed appearance of the maximum specific activity.

No information is available about the possible holdup of cholesterol in the intestinal mucosa, although this would seem unlikely in view of its rapid appearance in lymph (15). With respect to the possibility that newly absorbed cholesterol is initially removed from the blood, animal experiments show that injected colloidal suspensions of cholesterol resembling chylomicrons rapidly disappear from the circulation and that the cholesterol thus removed subsequently reappears in the plasma (17, 18). In addition, although cholesterol esterification during the process of intestinal absorption is well documented (15, 16, 19), there is no evidence that the esters thus formed are identical with those normally found in the circulation. The slow rise observed in the plasma free and ester specific activity curves can tentatively be ascribed to relatively rapid absorption of the sterol from the intestine, rapid removal of the newly absorbed sterol from the blood, followed by its subsequent reappearance in the circulation.

The absorption of the fed cholesterol-4-C^{14} varied from 52 to 86 per cent (Table VI) as determined by the amount that was excreted in the feces during the first four days. These values are higher than those reported for the absorption of one gram doses of tritium labeled cholesterol (13). With the exception of CHC-5, the subjects in this study received approximately 10 mg. of labeled

cholesterol and the efficiency of absorption may be related to the large difference in the amount fed. It should be noted that patient, CHC-5, who received one gram of cholesterol-*d* with the tracer dose of radioactive sterol showed the smallest absorption of cholesterol (52 per cent). The portion of the fecal radioactivity derived from cholesterol-4-C^{14} that had been absorbed, metabolized, and subsequently excreted, and that which had completely escaped absorption are not known. Since only 0.35 to 1.76 per cent of the cholesterol-4-C^{14} appeared in the urine, the kidney is a minor route for the excretion of the steroid nucleus; a considerable fraction of the urinary radioactivity is in the form of steroid hormone metabolites (9).

After the feeding of cholesterol, from 4 to 15 per cent was present in the circulation at the maximum specific activity of free cholesterol (Table VI). Without considering any other tissues, the human liver contains about 4.5 gm. of cholesterol in isotope equilibrium with plasma cholesterol (18) and an additional three grams of sterol are in the red cells, which after 24 to 36 hours are also in equilibrium with plasma free cholesterol (9). Thus approximately 8 to 33 per cent of the administered dose is present in the liver and circulation, thereby accounting for a significant fraction of the sterol absorbed from the diet.

The fact that no labeled carbon dioxide was detected after the administration of cholesterol-4-C^{14} shows that carbon-4 remained attached to the rest of the steroid nucleus. This suggests that the steroid nucleus is not degraded and is probably eliminated from the body in an intact form, either through the urine, feces or skin. This finding is in agreement with the results of Chaikoff and his colleagues (20) who have shown that the carbon-4 of cholesterol-4-C^{14} was not converted to carbon dioxide by the rat, although cholesterol labeled at C-26 can be oxidized with the appearance of $C^{14}O_2$ in the expired air.

Plasma cholesterol radioactivity curves

In all the patients who received labeled cholesterol, except CHT-1 with xanthoma tuberosum and CHC-6 with the nephrotic syndrome, the plasma cholesterol specific activity curves behave in the characteristic manner described. The data of Biggs and his co-workers (13) who fed tritium labeled cholesterol are essentially similar to those reported here except that the specific activity of total plasma cholesterol was measured rather than that of ester cholesterol. Since 60 to 75 per cent of plasma cholesterol is esterified, measurements of total cholesterol give a good approximation of the specific activity of the ester fraction. Warner (21) mentions that the specific activity of free cholesterol is higher than that of ester during the "period of active absorption."

The curves for plasma free and ester cholesterol derived from the diet decline at similar rates after reaching their maxima and are comparable to the behavior of plasma cholesterol synthesized in the body from acetate. The ester specific activity remains higher than that of the free because the free cholesterol is continually being diluted by non-labeled cholesterol from the diet or by endogenous synthesis. A more complete description of this process appears elsewhere (1). The behavior of the free and ester cholesterol shown in Figure 2, fits the criteria of Zilversmit, Entenman, Fishler, and Chaikoff (22) for the relationship between precursor (free) and product (ester). Inasmuch as free cholesterol was given, the free sterol must be the ultimate precursor of the plasma ester cholesterol.

The declining portion of the specific activity curves may be resolved into the component exponential rates listed in Table V. Since both distribution phenomena and biochemical reactions contribute to the processes which account for the decline in specific activity, no unique significance can be attached to these rates; however, they are useful in characterizing the curves.

The congruity of the specific activity curves derived from either C^{14} or tritium labeled acetate, as illustrated in Figure 1, shows that acetate labeled with either isotopes may be used for the study of cholesterol metabolism. It is also noteworthy that the plasma cholesterol of subject CHC-5 who received cholesterol-4-C^{14} and cholesterol-*d* simultaneously possessed a constant deuterium to C^{14} ratio at several points on the specific activity curve where measurements were made (Table III). This implies the identical

handling of cholesterol-4-C^{14} and deuterium-labeled cholesterol by the body.

If the rates describing the decline of plasma cholesterol derived from cholesterol-4-C^{14} are compared with those representing the decline of plasma cholesterol formed by endogenous synthesis from acetate-2-C^{14}, it will be noted that they are remarkably similar. This is illustrated graphically in Figures 3 and 4. Either labeled cholesterol or labeled acetate may be used interchangeably to trace the decay curve of plasma cholesterol, provided it is recognized that the early portions of the specific activity curves derived from these two tracer materials are different.

Studies in hypercholesterolemia

The altered relationship between free and ester cholesterol in the subject with xanthoma tuberosum (Figure 5) as compared with patients with xanthoma tendinosum or normocholesterolemic subjects has been observed previously (23). While there are no comparable studies in nephrosis, the similarity of the plasma cholesterol specific activity curves in xanthoma tuberosum with those of the nephrotic syndrome (Figure 6) should be emphasized. Since in both the xanthoma tuberosum and the nephrotic syndromes but, not in xanthoma tendinosum, the plasma appears lipemic and excessive amounts of neutral fat, phospholipids and the abnormal Sf 10-400 lipoproteins (24) are found, the higher initial specific activity of plasma ester cholesterol may perhaps be correlated with these findings which are characteristic of essential hyperlipemia in addition to hypercholesterolemia. Excessive esterification during absorption or a modified esterification process during lipoprotein synthesis by liver may be responsible for the abnormal relation between plasma free and ester cholesterol.

There are no findings in the data from the four patients with hypercholesterolemia that might aid in explaining the sustained elevation of the plasma cholesterol. The decline of the specific activity curves in these patients is not appreciably different from those of patients with normal cholesterol levels. Since the component exponential processes which form the decay curves are many in number, and have not been individually evaluated by any experimental procedure, small deviations that might be implicated in the maintenance of the elevated cholesterol levels may be "buried" in the curve. Small sustained differences in the rate of decay could bring about large cumulative changes in the plasma cholesterol level.

Interrelations of dietary and biosynthetic cholesterol

Dietary cholesterol and cholesterol made *in vivo* from acetate differ in metabolism only during the immediate post-absorptive and post-synthetic period in that biosynthetic cholesterol rapidly appears in the blood while the peak circulatory level of dietary cholesterol is delayed. However, the radioactivity data indicate conclusively that the two "species" of cholesterol merge by the third or fourth day following synthesis or ingestion. The fed, performed cholesterol is then indistinguishable from that synthesized from acetate except by application of isotope techniques. Since biosynthetic cholesterol is constantly secreted in the bile and mixes in the intestine with cholesterol present in food, a *mixture* of preformed and biosynthetic cholesterol is always available for absorption.

One difference between fed and biosynthesized cholesterol may be in the mode of transport immediately after either formation or absorption. It is likely that biosynthetic cholesterol is secreted into the circulation from the site of synthesis in combination with lipoprotein. The molecular form in which dietary cholesterol enters the circulation *via* the lymph is unknown and may differ in physical properties from lipoprotein-bound endogenous cholesterol. Since the physiological processes that give rise to both "species" are continuous, both forms are present in the circulation at the same time, and the possibility remains that the immediate post-absorptive phase of cholesterol metabolism is critical; further studies of these implications are in progress.

SUMMARY

1. Cholesterol and acetate, labeled with either radiocarbon or radiohydrogen, have been used to trace the metabolic behavior of plasma cholesterol synthesized *in vivo* or derived from the diet in

human subjects with normal and elevated plasma sterol levels.

2. From 4 to 27 per cent of a 10 mg. dose of labeled cholesterol appeared in the plasma after oral administration, 14 to 48 per cent was recovered in the feces during the first four days and approximately one per cent appeared in the urine. As suggested by the absence of measureable radioactivity in expired carbon dioxide, there was no evidence for the breakdown of the steroid nucleus.

3. Traces of orally administered cholesterol-4-C^{14} could be detected in the plasma within one hour and the maximum radioactivity of free and ester cholesterol was attained in two to three days. This is in contrast to labeled plasma cholesterol synthesized *in vivo* from radioactive acetate which reaches peak specific activity within eight hours.

4. No differences in the patterns of incorporation could be detected in two patients with hypercholesterolemia accompanying the xanthoma tendinosum syndrome when compared with subjects having normal sterol levels. Differences in the relation of free to ester cholesterol were observed during the incorporation of dietary cholesterol in a patient with hypercholesterolemia accompanying the xanthoma tuberosum syndrome and in another subject with hypercholesterolemia of the nephrotic syndrome.

5. Aside from early differences in the appearance of biosynthetic and dietary cholesterol in plasma cholesterol, the data suggest that cholesterol derived from the diet is eventually indistinguishably mixed with cholesterol synthesized in the body.

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MECHANISMS CONTROLLING CATION CONCENTRATIONS IN THE HUMAN CELL: EVIDENCE FROM THE EFFECT OF IODOACETATE ON Na AND K EXCHANGE RATES OF THE ERYTHROCYTE [1]

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The mechanisms controlling intracellular electrolyte content are of clinical interest because abnormalities of intracellular electrolyte distribution occur in a variety of illnesses. Tissue electrolyte metabolism can be investigated by the direct study of muscle removed by biopsy, or by the use of balance technics from which deductions about changes in the quantity of intracellular electrolyte are made. However, neither of these methods is suited for extensive clinical application. Therefore, the readily obtainable circulating erythrocytes (RBC) have been studied with the hope that they would prove to be sufficiently similar to the bulk of body cells in their metabolism to reflect the intracellular electrolyte state of the body as a whole. Reports of abnormalities in the Na and K content of RBC in association with disease have appeared (1–4), but proof of a parallel relationship between the changes in RBC and those in other cells is not yet available. Like other human cells, the erythrocytes of man contain a high concentration of potassium and a low concentration of sodium, despite the continuous exchange of both of these cations between the cells and plasma (5–11). It has been maintained that active mechanisms exist in the erythrocyte for the inward transport of potassium and for the expulsion of sodium against the concentration gradients and that these processes are energized by glycolysis (11–13). The experiments reported herein were conducted to test this hypothesis.

Iodoacetate, which is known to abolish glycolysis and selective cation accumulation in erythrocytes (14), was added to human whole blood maintained under simulated physiologic conditions *in vitro*. By the use of Na^{22}, K^{42}, and Rb^{86}, the changes in the rates with which Na and K entered and left the RBC were measured. If iodoacetate abolished the active processes postulated to exist in the RBC, the expected result would be decreases in the rates of entry of potassium and exit of sodium.

METHODS

The methods employed in these studies have been described in detail elsewhere (8–10). Heparinized human blood was placed in paraffined flasks and gently agitated at 38° C. Na-mono-iodoacetate in a final concentration of 10^{-3} M was added fifteen minutes before the isotopes. The rate at which sodium entered the RBC was measured by adding Na^{22} to the plasma and following the rise of specific activity in the erythrocytes with time. The rate of return of sodium from the erythrocytes to the plasma was considered to be equal to the difference between the rate of entry of sodium into the cells and the rate of change in total sodium content of the erythrocytes. In three experiments Rb^{86} was used to trace potassium by the method discussed elsewhere (10). Rb^{86} was shown to be utilized by normal RBC *in vitro* in a manner that is quantitatively and qualitatively very similar to that of potassium. In fact, by the methods used, no difference in their behavior in normal cells was detected. Figure 1 shows that this similarity of behavior is also present in RBC under the influence of iodoacetate. By measuring changes in "specific activity" (Rb^{86} cpm. per mEq. K^{39} as well as K^{42} cpm. per mEq. K^{39}) of plasma and erythrocytes, the rates of potassium intake and output by the RBC were both observed directly. The radioactivity was measured with a thin, mica end-window G. M. tube. Sodium and potassium were determined with a modified Beckman model D. U. flame photometer, making empirical corrections for mutual excitation of sodium and potassium. The erythrocytes were separated from the plasma by means of a special clamp which allowed RBC centrifuged in plastic tubes to be isolated with an estimated plasma content of 1.9 per cent (8). Serial determinations of hematocrit in three experiments revealed no significant change in RBC volume up to seven hours after adding iodoacetate.

Calculations of exchange rate were made by the method of Sheppard and Martin (5), assuming that RBC and

[1] Aided by a Public Health Service grant H-143, U. S. A. E. C. Grant No. AT-(40-1)-233, and the Mrs. E. J. Caire Fund for Research in Heart Disease.

plasma form two uniformly mixed communicating compartments. Since the rates of transfer into and out of the RBC were not equal and the contents of the compartments were changing in a reciprocal fashion, rates were calculated from hour to hour using the following formulae of Sheppard:

$$\frac{dS_{21}}{dt} = \frac{S_2 \cdot \frac{da_2}{dt}}{a_1 - a_2}$$

$$\frac{dS_{12}}{dt} = \frac{S_1 \cdot \frac{da_1}{dt}}{a_2 - a_1}$$

where

$\frac{dS_{21}}{dt}$ and $\frac{dS_{12}}{dt}$ are respectively the rates of transfer of nontracer material into and out of the RBC,

S_1 and S_2 are the total nontracer sodium or potassium content of the plasma and RBC respectively, and

a_1 and a_2 are the specific activities of plasma and RBC respectively (Na^{22}/Na^{23}, Rb^{86}/K^{39}, or K^{42}/K^{39}).

$\frac{da_1}{dt}$ and $\frac{da_2}{dt}$ were determined graphically from observed data.

Since approximately two-thirds of the RBC sodium exchanges with the plasma in one hour, RBC sodium specific activity rapidly approached that of the plasma. When the difference between their specific activities became small, small errors in the determination of specific activity in either compartment made large percentile errors in the absolute difference between them. Since these errors were not necessarily random ones that would be subject to statistical treatment, calculations of exchange rate have not been reported when RBC specific activity had reached three-fourths of the plasma specific activity.

RESULTS

Changes in RBC Na and K concentrations after addition of iodoacetate

In each experiment there was a nearly linear rise in RBC sodium content after addition of iodoacetate to the whole blood, the mean rate of increase in RBC sodium concentration being 1.18 mEq. Na per L. RBC per hr. (Figure 2b). The rate of fall in RBC potassium was greater than the rate of gain of sodium, averaging 1.80 mEq. K per L. RBC per hr. when measured directly (Figure 2c) and 1.67 mEq. K per L. RBC per hr. when determined from the rise in plasma potassium, which is expressed as mEq. per L. RBC present in the whole blood (Figure 2a). These net transfers of sodium and potassium began in less than one hour after adding iodoacetate.

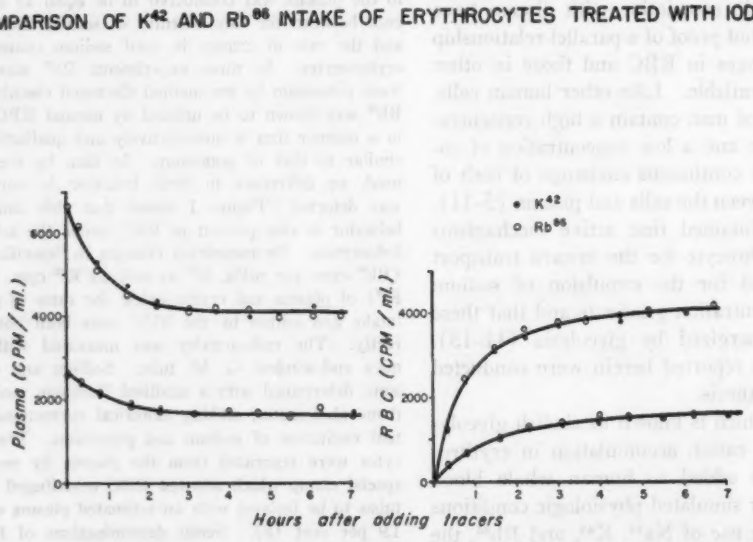

FIG. 1. COMPARISON OF THE INTAKE OF K^{42} AND Rb^{86} BY RBC IN TWO SPECIMENS OF HUMAN WHOLE BLOOD AT 38° C. *In Vitro*

Iodoacetate (10^{-3} M) was added 15 minutes before the isotopes. The differences in counting levels are caused by the addition of unequal amounts of tracers in the two instances.

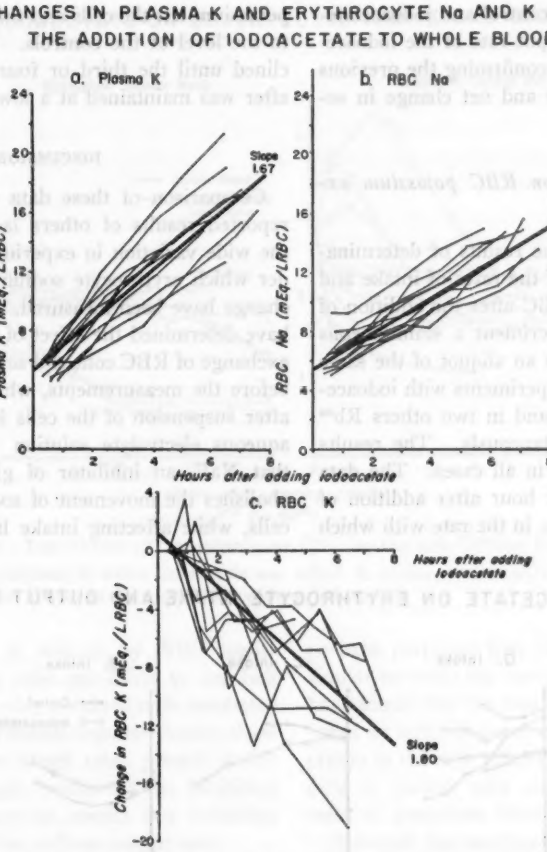
CHANGES IN PLASMA K AND ERYTHROCYTE Na AND K AFTER THE ADDITION OF IODOACETATE TO WHOLE BLOOD
a. Plasma K
Plasma K (mEq./L.RBC)
Slope 1.67
b. RBC Na
RBC Na (mEq./L.RBC)
Slope 1.18
Hours after adding iodoacetate
c. RBC K
Change in RBC K (mEq./L.RBC)
Hours after adding iodoacetate
Slope 1.80

FIG. 2. THE TIME COURSE OF THE RISE IN PLASMA POTASSIUM (a) AND ERYTHROCYTE SODIUM (b) AFTER THE ADDITION OF IODOACETATE (10^{-4} M) TO HUMAN WHOLE BLOOD AT 38° C. *In Vitro*

The fall in RBC potassium is shown in (c). Plasma potassium is expressed as mEq. per L. RBC. The regression lines calculated by the method of least squares are indicated.

Influence of iodoacetate on RBC sodium exchange rates

In Figure 3a the rate of intake of sodium by normal cells in three instances is compared to the rate of intake by the cells in two determinations in which iodoacetate was added. Figures 3b, c, and d show the same comparison made from simultaneous determinations on aliquots of the same blood samples. There is no evidence in these data of an increase in the rate of intake of sodium after the addition of iodoacetate. Therefore, since the sodium content of the iodoacetate-treated cells was increasing at an average rate of 1.18 mEq. Na per L. RBC per hr., the rate of output of sodium from them must have been less than in the controls. In the experiment illustrated in Figure 3d, three and one-half hours after the addition of Na^{22} the blood was lightly centrifuged and the plasma was replaced with tracer-free plasma. This allowed the direct determination of the rates of both intake and output of sodium by these cells as the Na^{22} flowed out of them. These rates are indicated in Figures 3e and f. Again the

intake rate was similar in control and iodoacetate-treated blood; and the output rate of the iodoacetate-treated cells was low, confirming the previous inference from intake rate and net change in sodium content.

Influence of iodoacetate on RBC potassium exchange rates

Figure 4 summarizes the results of determinations of the time course of the rates of intake and output of potassium by RBC after the addition of iodoacetate. In each experiment a simultaneous control was carried out on an aliquot of the same blood sample. In three experiments with iodoacetate Rb^{86} was the tracer, and in two others Rb^{86} and K^{42} were used simultaneously. The results were essentially the same in all cases. The data show that during the first hour after addition of iodoacetate there was a rise in the rate with which potassium left the cells and that later this returned to the level of the controls. The intake rate declined until the third or fourth hour, and thereafter was maintained at a low rate.

DISCUSSION

Comparison of these data with the previously reported results of others is difficult because of the wide variation in experimental conditions under which erythrocyte sodium and potassium exchange have been measured. Harris and Maizels have determined the effect of NaF on the sodium exchange of RBC collected several hours or longer before the measurements, which were carried out after suspension of the cells in a large volume of aqueous electrolyte solution (15). They found that NaF, an inhibitor of glycolysis, essentially abolishes the movement of sodium out of the red cells, while affecting intake little. However, the

EFFECT OF IODOACETATE ON ERYTHROCYTE INTAKE AND OUTPUT RATES OF Na

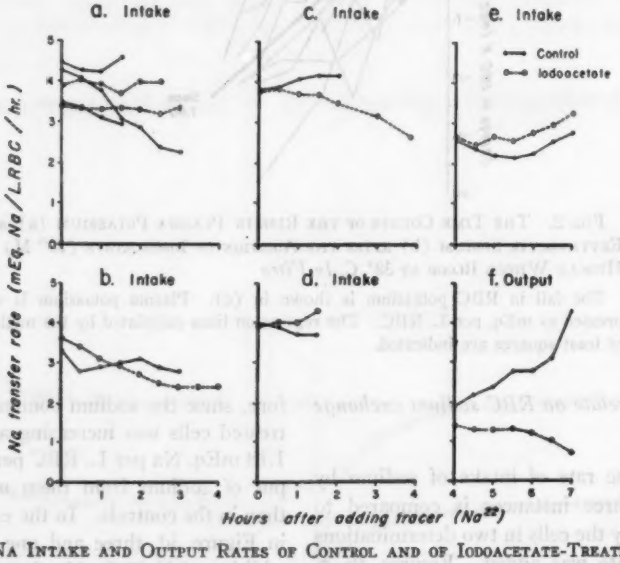

FIG. 3. NA INTAKE AND OUTPUT RATES OF CONTROL AND OF IODOACETATE-TREATED RBC

a. Na intake in three controls compared with that in two measurements on iodoacetate-treated cells.

b, c, and d. Simultaneous measurements of Na intake in control and iodoacetate-treated cells from the same blood samples.

e and f. Na intake and output rates of the cells illustrated in (d), measured from four to seven hours after adding Na^{22}. The original plasma was replaced by tracer-free plasma after three and one-half hours of incubation.

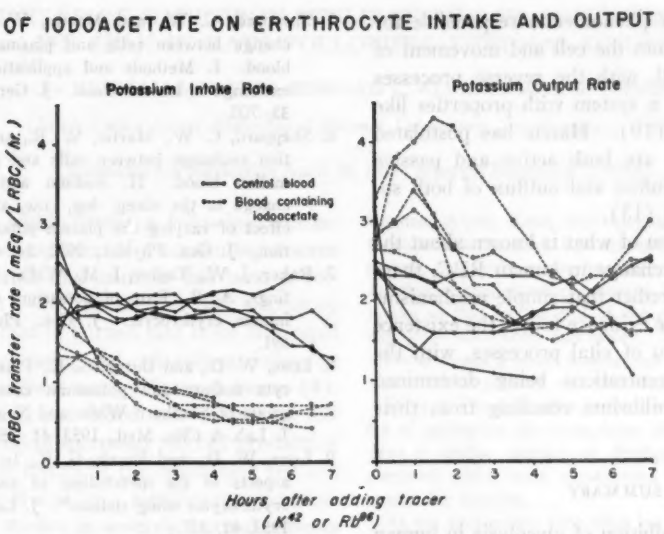
EFFECT OF IODOACETATE ON ERYTHROCYTE INTAKE AND OUTPUT RATES OF K
Potassium Intake Rate
Potassium Output Rate
RBC transfer rate (mEq./L.RBC/hr.)
Control blood
Blood containing iodoacetate
Hours after adding tracer
(K42 or Rb86)

FIG. 4. THE EFFECT OF IODOACETATE ON RBC INTAKE AND OUTPUT RATES OF K

In each experiment in which iodoacetate was added, K exchange rates were measured simultaneously on an aliquot of the same blood without iodoacetate.

fractional exchange of sodium by RBC under these conditions was only one-third to one-half of that observed in freshly obtained cells incubated in plasma. The experiments reported herein show that at the higher exchange rates present under more nearly physiologic conditions the inhibition of glycolysis by iodoacetate results in a reduction of only one-third in the sodium output rate.

Taylor, Weller, and Hastings have reported measurements of K exchange rates in the presence of NaF (16). In their method fresh cells are incubated in an inorganic medium containing glucose. Under these conditions the K exchange rate was essentially the same as that observed in plasma. The initial entry rate was determined from the initial slope of the curve of loss of "plasma" K^{42}. The exit rate was equal to the sum of the initial entry rate and the rate of net loss of RBC potassium. They reported that the rate of entry was decreased by 60 per cent and the rate of exit increased by 75 per cent. Eckel, in experiments carried out by similar technics, has likewise reported an accelerated output rate, although the increase was six to twelve times the normal rate (17). However, in his results an increase in the intake rate proportional to the rise in concentration of plasma K was present. It is perhaps pertinent that Sheppard and Beyl, using similar methods but incubating the cells in plasma, have found that the loss of potassium by RBC injured by large doses of x-rays is caused by an increase in the rate of movement of potassium from cells to plasma with essentially unaltered movement of potassium from plasma to cells (18).

Solomon has analyzed the processes responsible for sodium and potassium exchange in the RBC from the thermodynamic viewpoint (11). He considers that the available data show that sodium and potassium are transported into the cell by different mechanisms, that the processes responsible for the entry and exit of sodium are different, and that potassium influx and outflux are probably not affected by the same process either. This latter conclusion is supported by the report of Taylor, Weller, and Hastings that cholinesterase inhibitors cause loss of RBC potassium by decreasing the intake rate, whereas choline acetylase inhibitors cause a loss of potassium by increasing the output rates (16). Solomon concludes that in RBC neither sodium nor potassium is transported in either direction by a process like simple diffusion. However, Cooke and Segar have recently postulated a mechanism for controlling muscle electrolyte composition which involves pas-

sive diffusion as the phenomenon responsible for loss of potassium from the cell and movement of sodium into the cell, with the reverse processes being performed by a system with properties like an exchange resin (19). Harris has postulated that in RBC there are both active and passive components of the influx and outflux of both sodium and potassium (13).

From consideration of what is known about the process of cation exchange in human RBC, there is little reason to predict that simple mechanisms are responsible. The evidence favors the existence of a complex system of vital processes, with the cellular cation concentrations being determined by the dynamic equilibrium resulting from their interaction.

SUMMARY

Following the inhibition of glycolysis in human erythrocytes by Na-mono-iodoacetate there was a net flow of sodium into the cells (1.18 mEq. Na per L. RBC per hr.) and a greater flow of potassium out of them (1.80 mEq. K per L. RBC per hr.). Determination of sodium and potassium exchange rates by the use of radioisotopes showed that the net gain of sodium was caused by a decrease in sodium output with the intake being little affected; whereas, the loss of potassium was caused by both an increase in output and a decrease in intake.

These results confirm the presence of active governing processes in RBC which promote the intake of potassium and the output of sodium and inhibit the output of potassium.

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STUDIES OF RADIOIRON UTILIZATION AND ERYTHROCYTE LIFE SPAN IN RATS FOLLOWING THERMAL INJURY [1]

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The anemia which develops following thermal burns and the occurrence of a continuing anemia in burned patients and animals has been a frequent finding (1–4). Studies in this laboratory have indicated that in burned rats there is a rapid reduction in circulating red cell volume following high intensity radiant energy thermal burns (4). This reduction in red blood cell volume is followed by a period of increased red cell production as shown by the return of the circulating red cell volume to normal and by a significant reticulocytosis. Clinical studies in severely burned patients (2, 3) have revealed evidence of an immediate reduction in the circulating red cell volume and frequently a continuing reduction of the red cell volume.

The studies of Moore, Peacock, Blakely, and Cope (1) in three burned patients and of Wintrobe and his co-workers (5) in two burned pigs showing depressed utilization of radioiron are the only reports of the role of iron metabolism in burn anemia. Reported here are measurements of utilization of tracer doses of radioiron (Fe^{59}) in the production of erythrocytes, and measurements of erythrocyte life span with Fe^{59}.

METHODS

Six-week-old female albino rats of the Sprague-Dawley strain from the Laboratory colony were used. These rats weighed between 140 and 180 gm. at the time of burning. They were housed in individual wire cages and allowed free access to Purina chow and water. All animals were depilated by the method of Kuhl, Sheline, and Alpen (6). They were anesthetized with Nembutal® sodium (4.5 mg. per 100 gm. body weight subcutaneously) 4 days later and burned with a high intensity carbon arc source (7) by a method previously described. The burns produced were approximately 25 per cent of the total body area, and were similar, grossly and histologically, to the "white" burn described by Sheline, Alpen, Kuhl, and Ahokas (7). After two to three weeks the gray leathery eschar separated, leaving a raw, relatively clean, partially re-epithelialized area. Further epithelialization occurred in the next six to ten weeks so that by the end of the observation period (90 days) the burn areas had healed. Superficial infection of the lesion was a usual finding, but no significant surface loss of blood or fluid was observed.

Days 2, 7 and 15 after burning were selected for studies of utilization of tracer doses of radioiron. On these days a similar number of depilated control rats were prepared which were anesthetized and similarly treated except for burning.

At the appropriate time after burning, each animal was given a standard dose of 0.6 ml. of a sodium citrate buffered $FeCl_3$ solution, which contained 3 to 10 μc. of Fe^{59} in approximately 6 to 20 μg. of total iron. The amount of radioactivity and the specific activity of the injected iron was the same for each group but varied among the various experimental trials. The Fe^{59} used was $Fe^{59}Cl_3$ obtained from Oak Ridge National Laboratory and contained less than 10 per cent of Fe^{55}. The tracer dose was injected into the surgically exposed external jugular sinus. The surgical wound was closed with skin clips and healed promptly. Blood samples were taken at six hours and days 1, 2, 3, 5, and 7 following injection of the tracer and at weekly intervals thereafter. Blood samples were drawn by cardiac puncture or external jugular vein puncture through the intact skin. Samples of approximately 0.3 ml. of whole blood were taken during the first week; thereafter the samples were 0.1 to 0.2 ml. of whole blood. Twenty-five μl. of whole blood was plated on cupped planchets in duplicate. Plasma was obtained by centrifugation of blood immediately after withdrawal of the blood, and 25 μl. amounts were plated in duplicate on planchets. Hematocrits were performed in duplicate in thick-walled, uniform-bore capillary tubes, sealed with mercury and beeswax and spun for 30 minutes at 3000 RPM in a refrigerated centrifuge at 5° to 10°C. The packed red cell and plasma columns were read with a 10 cm. scale.

Radioactivity was measured with an end window scintillation counter. An automatic sample changer, scaler, and automatic timing device were used, and each set of duplicate planchets was counted to a counting error of 0.5 per cent. The over-all counting efficiency was approximately 4 per cent. At the point of maximum utilization of the injected Fe^{59}, 25 μl. samples gave counting rates approximately 10 to 50 times background. At the conclusion of the experiment when circulating radio-

[1] The opinions or assertions contained herein are those of the writers and are not to be construed as official or reflecting the views of the Navy Department or the Naval Establishment at large.

activity had stabilized at minimal values, counting rates (50 or 100 μl. samples) were approximately 2 to 5 times background.

Radioactivity of the total circulating whole blood was calculated using an estimated blood volume. A large body of observations have been made in this laboratory using the P^{32} method (4) in both control and burned rats of similar age and strain. These values were used for blood volume calculated on a weight basis for given days after burning. Red cell volumes and plasma volumes were calculated using the hematocrit determined from jugular or cardiac blood. Total circulating red cell radioactivity was obtained by subtracting total circulating plasma radioactivity from total circulating whole blood radioactivity. It was found that after 24 hours the plasma had been cleared of essentially all Fe^{59} in both control and burned animals and that the measured radioactivity was confined to the circulating red cells. For this reason only whole blood samples were counted beyond the first week. Since with a given measured tracer injection dose an identical measured injection dose standard was prepared and was counted simultaneously with the blood samples, it was unnecessary to correct for decay of radioactivity. However, because of multiple blood sampling of each animal it was necessary to correct for removed radioactivity.

The same animals were kept under observation and utilized for studies of mean erythrocyte life span by the method of Finch, Wolff, Rath, and Fluharty (8) and Burwell, Brickley, and Finch (9). Starting on the seventh day after injection of the tracer dose of radioiron, each rat was given a weekly intravenous injection of 0.4 ml. of non radioactive saccharated iron oxide containing 8 mg. of inorganic iron. Weekly injections were continued for 10 weeks, at which time circulating radioactivity had stabilized at minimal levels in each animal. A total of 80 mg. of iron was given to each animal. In the studies of erythrocyte life span weekly samples were drawn by cardiac or external jugular puncture just prior to each weekly intravenous injection of saccharated iron oxide. Sampling was continued until circulating radioactivity had stabilized (usually between 5 and 15 per cent of the injected radioactivity). A separate control group of five animals, identical except that the weekly intravenous injections of saccharated iron oxide were omitted, was also followed. This group was studied for the purpose of ascertaining the extent to which iron loading inhibits the reutilization of Fe^{59}.

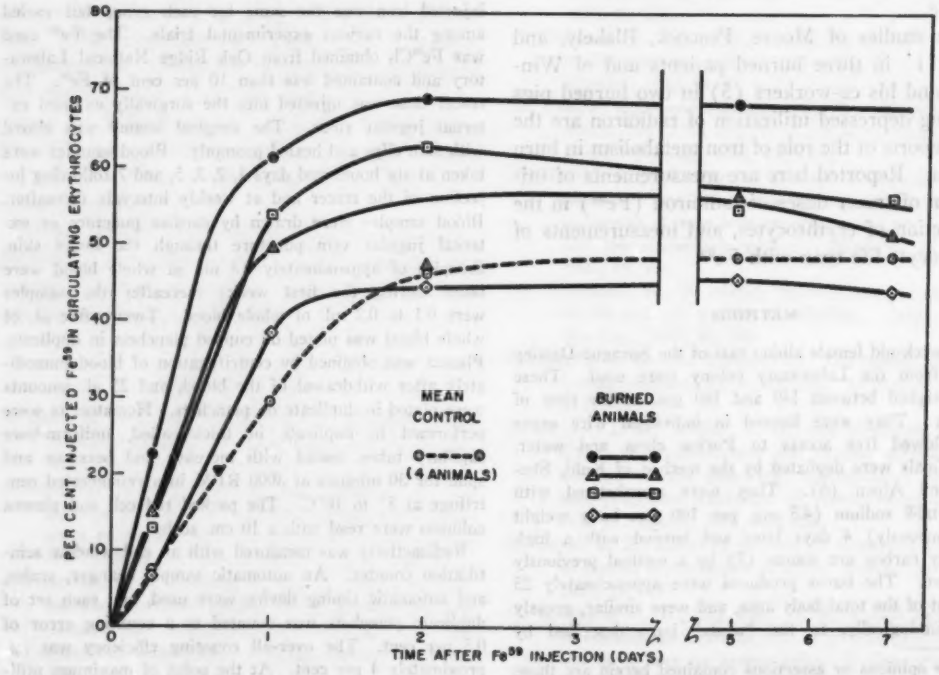

FIG. 1. UTILIZATION OF Fe^{59} IN FOUR BURNED ANIMALS INJECTED WITH Fe^{59} TWO DAYS AFTER BURNING, COMPARED WITH THE MEAN UPTAKE OF Fe^{59} FOR FOUR CONTROL ANIMALS RUN SIMULTANEOUSLY

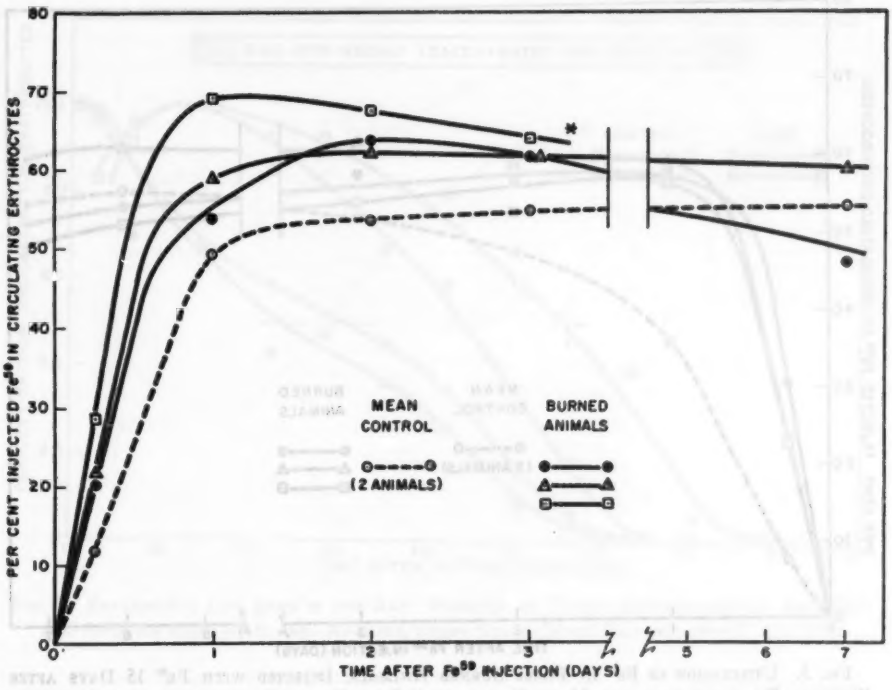
PER CENT INJECTED Fe59 IN CIRCULATING ERYTHROCYTES
TIME AFTER Fe59 INJECTION (DAYS)
MEAN CONTROL (2 ANIMALS)
BURNED ANIMALS

FIG. 2. UTILIZATION OF Fe^{59} IN THREE BURNED ANIMALS INJECTED WITH Fe^{59} SEVEN DAYS AFTER BURNING, COMPARED WITH THE MEAN UPTAKE OF Fe^{59} FOR TWO CONTROL ANIMALS RUN SIMULTANEOUSLY

RESULTS

Utilization of tracer doses of Fe^{59}

Figures 1, 2, and 3 indicate the utilization of Fe^{59} in control and burned rats for seven days following injection of a tracer dose of Fe^{59}. The rate of uptake of Fe^{59} in burned rats was studied commencing on the second, seventh, and fifteenth day following burning. The uptake was found to be more rapid and the fraction of the injected dose of radioiron appearing in red cells was greater than in control animals at 24, 48, and 72 hours after injection of the radioisotope. Moreover, for the 7-day and 15-day groups at the end of one week, the per cent incorporation in the erythrocytes of both control and burned rats tended to approach a common value in the range of 50 to 60 per cent. The group of burned animals studied two days after burning showed a somewhat greater variation in uptake than animals studied at later times after burning. However, the per cent of the injected dose incorporated was still higher than control values at one week after injection.

Measurement of mean erythrocyte life span

In Figures 4, 5, and 6 are plotted curves of the total radioactivity in red cells of peripheral blood remaining at various times following initial uptake of Fe^{59}. These figures are for studies of survival started with injection of Fe^{59} on days 2, 7, and 15 after burning, respectively. The mean erythrocyte life span is estimated by measuring the time in days required for the total radioactivity to decrease to one-half its maximum value. It is necessary in these calculations to correct for the 5 to 15 per cent of radioactivity remaining at the stabilized low level eventually reached (Figure 7). The data are therefore plotted by subtracting the stable residual activity from the measured value and plotting these corrected values in terms of per cent of the maximum radioactivity attained.

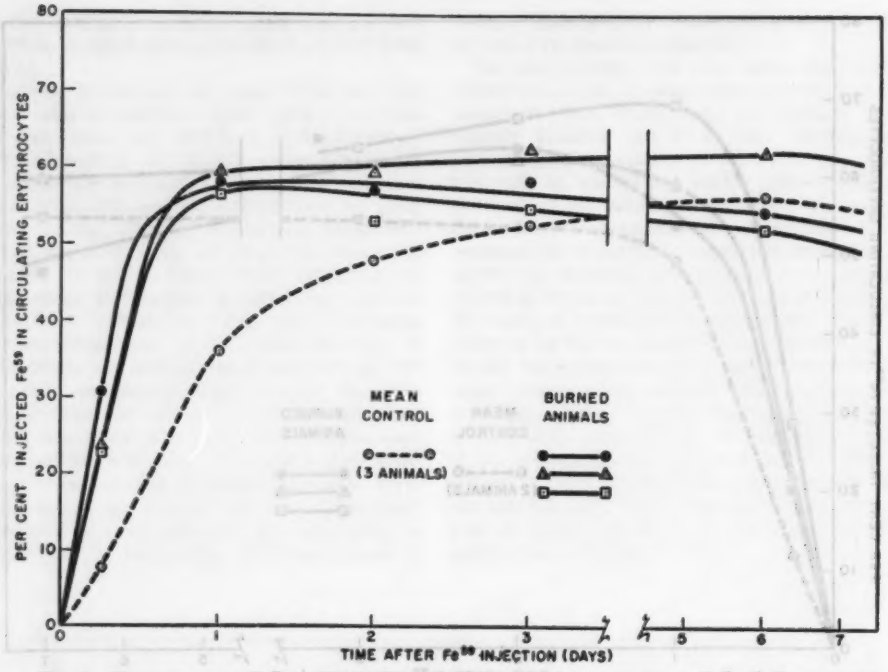
PER CENT INJECTED Fe59 IN CIRCULATING ERYTHROCYTES
TIME AFTER Fe59 INJECTION (DAYS)
MEAN CONTROL (3 ANIMALS)
BURNED ANIMALS

FIG. 3. UTILIZATION OF Fe^{59} IN THREE BURNED ANIMALS, INJECTED WITH Fe^{59} 15 DAYS AFTER BURNING, COMPARED WITH THE MEAN UPTAKE OF Fe^{59} FOR THREE CONTROL ANIMALS RUN SIMULTANEOUSLY

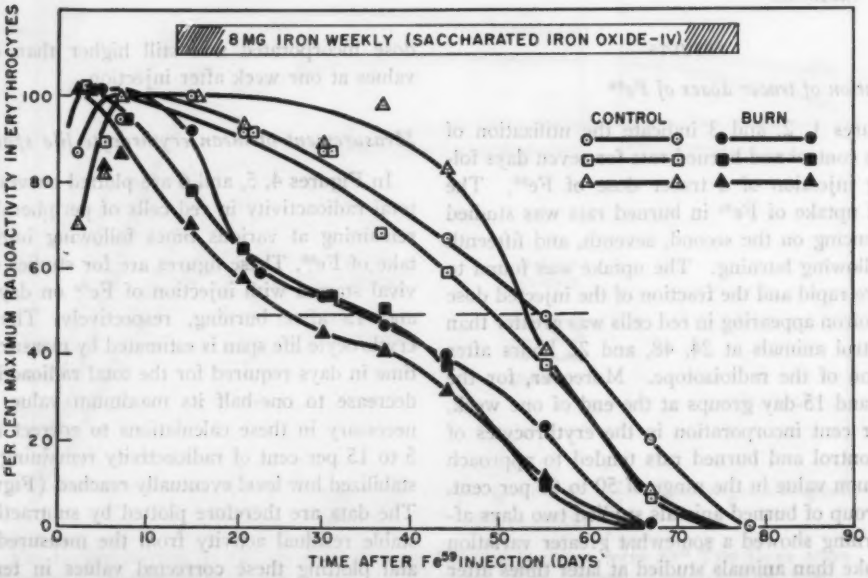
8 MG IRON WEEKLY (SACCHARATED IRON OXIDE-IV)
PER CENT MAXIMUM RADIOACTIVITY IN ERYTHROCYTES
TIME AFTER Fe59 INJECTION (DAYS)
CONTROL
BURN

FIG. 4. ERYTHROCYTE LIFE SPAN IN THE RAT. SURVIVAL OF TAGGED ERYTHROCYTES IN THREE CONTROL ANIMALS AND THREE BURNED ANIMALS, STUDY BEGUN TWO DAYS FOLLOWING THE BURN

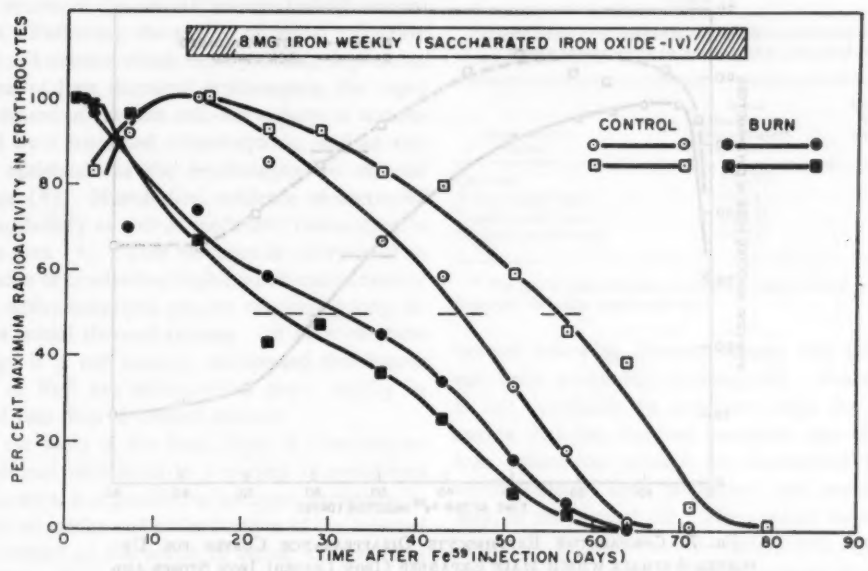
8 MG IRON WEEKLY (SACCHARATED IRON OXIDE-IV)
CONTROL
BURN
PER CENT MAXIMUM RADIOACTIVITY IN ERYTHROCYTES
TIME AFTER Fe59 INJECTION (DAYS)

FIG. 5. ERYTHROCYTE LIFE SPAN OF THE RAT. SURVIVAL OF TAGGED ERYTHROCYTES IN TWO CONTROL ANIMALS AND TWO BURNED ANIMALS, STUDY BEGUN SEVEN DAYS FOLLOWING THE BURN

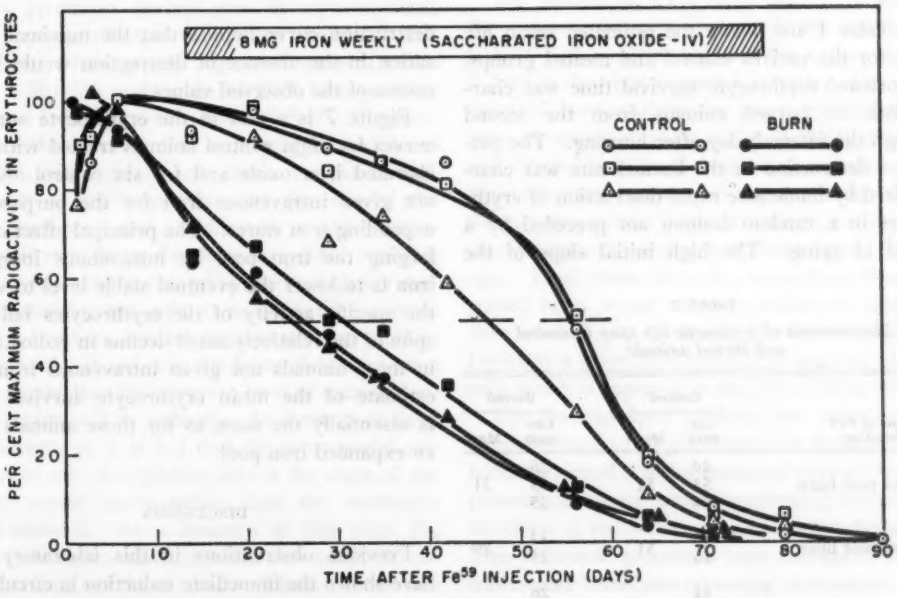
8 MG IRON WEEKLY (SACCHARATED IRON OXIDE-IV)
CONTROL
BURN
PER CENT MAXIMUM RADIOACTIVITY IN ERYTHROCYTES
TIME AFTER Fe59 INJECTION (DAYS)

FIG. 6. ERYTHROCYTE LIFE SPAN OF THE RAT. SURVIVAL OF TAGGED ERYTHROCYTES IN THREE CONTROL ANIMALS AND THREE BURNED ANIMALS, STUDY BEGUN 15 DAYS FOLLOWING THE BURN

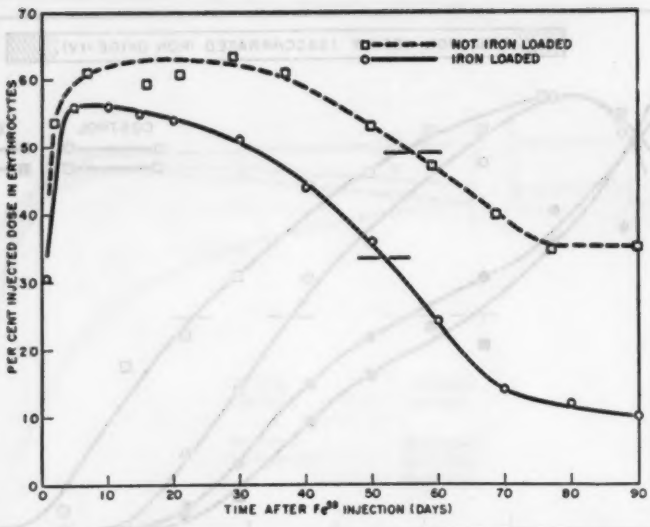

FIG. 7. COMPARATIVE ERYTHROCYTE DISAPPEARANCE CURVES FOR UNBURNED ANIMALS WHICH HAVE EXPANDED (IRON LOADED) IRON STORES AND NORMAL (NOT IRON LOADED) IRON STORES

The horizontal dash indicates the points of 50 per cent destruction as calculated from the point of maximum uptake to the point of lowest stable radioactivity. The "not iron loaded" curve represents the mean of six animals while the "iron loaded" curve represents the mean of eight animals.

In Table I are shown the estimated mean life spans for the various control and treated groups. A shortened erythrocyte survival time was characteristic of burned animals from the second through the fifteenth day after burning. The pattern of destruction in the burned rats was characterized by immediate rapid destruction of erythrocytes in a random fashion not preceded by a period of aging. The high initial slope of the destruction curve implies that the maximal utilization in the absence of destruction would be in excess of the observed values.

TABLE I

Measurement of erythrocyte life span in control and burned animals

Time of Fe^{59} injection	Control		Burned	
	Life span	Mean	Life span	Mean
2 days post burn	49	51	26	31
	53		33	
	54		35	
7 days post burn	56	51	33	29
	46		25	
15 days post burn	54	51	26	25
	54		25	
	45		24	

Figure 7 is a plot of the erythrocyte survival curves for eight control animals treated with saccharated iron oxide and for six control animals not given intravenous iron for the purposes of expanding iron stores. The principal effect of enlarging the iron pool by intravenous inorganic iron is to lower the eventual stable level to which the specific activity of the erythrocytes fall. In spite of the relatively small decline in radioactivity in those animals not given intravenous iron, the estimate of the mean erythrocyte survival time is essentially the same as for those animals with an expanded iron pool.

DISCUSSION

Previous observations in this laboratory (4) have shown the immediate reduction in circulating red cell volume following high intensity radiant energy thermal burns in the rat, and the subse-

quent return of the red cell volume toward normal values. Following the period of initial reduction in red cell volume which most probably represents removal of heat damaged erythrocytes, the rapid replacement of the lost red cell volume is accompanied by a sustained reticulocytosis, and an elevated resistance of the erythrocytes to osmotic damage (4). Histological evidence of increased extramedullary as well as medullary hematopoiesis is also seen (4). This evidence is interpreted as indicative of accelerated replacement compensating for an active hemolytic process continuing long after the initial thermal trauma. In view of these findings it is not entirely unexpected that tracer doses of Fe^{59} are incorporated more rapidly in burned rats than in control animals.

On the basis of the large body of observations of radioiron utilization in a variety of conditions and diseases, it is possible to interpret variations in the rate of uptake and in the fraction of the injected dose utilized as the result of either altered rates of erythropoiesis (10) or change in the size of the available iron pool. Finch, Wolff, Rath, and Fluharty (8) have demonstrated the decreased level of radioiron utilization in hemochromatosis characterized by much enlarged stores of iron but a presumed normal rate of erythropoiesis. The opposite effect is seen in the anemia of iron deficiency where greatly augmented rates of radioiron utilization and levels of Fe^{59} incorporation are seen following tracer doses of Fe^{59}. The fraction of the injected dose appearing in peripheral erythrocytes depends on three variables: (a) the rate of erythropoiesis; (b) the size of the labile iron pool; and (c) the rate at which radioactive iron is being deposited in poorly available iron stores. In the case of animals which are rapidly destroying red cells, as in the burned animals reported on here, another important factor must be considered. If red cells are being destroyed soon after production the apparent level of maximal incorporation will be reduced. As a result of these considerations, it is felt that the most useful criterion of rate of erythropoiesis is the slope of the initial uptake curve rather than the maximum level attained. As a measure of this slope the time required to reach one-half the maximum incorporation has been used. These values are shown in Table II. Although there is no *a priori* evidence that iron stores deviate greatly from normal following thermal injury, this point has not been adequately investigated. However, it is felt justifiable to conclude from the uptake curves that the observed increased rate of radioiron utilization reflects an accelerated rate of erythropoiesis, both medullary and extramedullary, in the burned rat. The greater variation in uptake in burned animals studied two days after the burn compared with the animals studied at later times after burning indicates that the accelerated rate of erythropoiesis occurring as a result of the burn is still a variable finding at this early time.

TABLE II

Relative rates and amounts of Fe^{59} incorporation in circulating erythrocytes in burned and untreated rats

Experimental group	Mean maximum incorporation (*per cent*)	Time for half maximum incorporation (*days*)
Control	54 (9)*	0.76
2 days post burn	58 (4)	0.49
7 days post burn	65 (3)	0.37
15 days post burn	59 (3)	0.33

* Figure in parentheses indicates the number of animals included in each mean value.

The studies of erythrocyte survival show that the mean erythrocyte life span in the burned rats is 27 ± 4 days compared to mean values of 51 ± 5 days in the normal control animals. The values reported here confirm the mean erythrocyte life span of normal rats found by Burwell, Brickley, and Finch (9) to be 45 to 50 days, utilizing essentially the same method. They emphasize that in the rat the removal of red cells is essentially a random process occurring without regard for cell age. Their data, however, as well as those reported here, would seem to indicate a relatively slow random process during the first 30 days followed by a more rapid process of removal of older cells which is apparent in the period 30 to 60 days.

The findings here reported are interpreted to indicate that in rats the anemia of thermal injury is characterized both by increased rates of erythropoiesis and an accelerated continuing random destruction of red blood cells, *i.e.*, active hemolysis. This hemolytic process is over and above the intravascular hemolysis occurring as an acute event during or immediately after the thermal injury itself.

Moore, Peacock, Blakely, and Cope (1) report six measurements of radioiron utilization at scattered points in three severely burned human patients. In the first patient iron utilization measured at one month and three months following the burn was still depressed. In a second patient, measured on the first day after the burn, Fe^{55} uptake was severely depressed but two utilization measurements made several months later in the course of the patient's recovery were essentially normal. Iron uptake in the third patient on the fifth day of a massive burn, which proved fatal, was normal. Wintrobe, Greenberg, Humphreys, Ashenbrucker, Worth, and Kramer (5), in a study of the anemia of infection and inflammation in iron deficient pigs, report radioiron utilization in two burned animals. In one animal the radioiron uptake was greatly depressed below the preinjury determination; in the other animal the uptake was slightly greater than the preinjury utilization. No studies have been undertaken in pig or man to follow erythrocyte survival subsequent to the uptake period.

The group at the Medical College of Virginia (2, 3) has recently reported on the utilization of N^{15} glycine for red cell production in burned dogs and patients. They have concluded that the anemia of thermal burns is the result of an early hemolytic process followed by a period of dyshematopoiesis. Objections against this interpretation are that the fecal urobilinogen as well as the hemolytic index remained elevated for many days following injury in severely burned patients. This may be construed as evidence of a continuing hemolytic process. In addition it is entirely possible that depression in production of a remote precursor to hemoglobin might exist without depression of red cell formation. Tissue stores of the precursor could be used until exhausted.

SUMMARY

The anemia of thermal injury has been investigated in rats subjected to high intensity radiant energy thermal burns. Observations of Fe^{59} utilization following injection of tracer doses have been made in control and burned animals 2, 7, and 15 days after burning and indicate an accelerated rate of Fe^{59} uptake in burned animals. Utilizing heavy iron loading after the period of uptake to depress reutilization, these same labeled erythrocytes have been used for measurement of erythrocyte life span in the same control and burned animals. The mean erythrocyte life span of normal rats has been found to be 51 days; the mean erythrocyte life span of burned animals has been measured as 27 days. The burned animal shows early and continuing random destruction of erythrocytes at a rate greater than normal. This hemolytic process is compensated for in the rat by increased rates of medullary and extramedullary hematopoiesis and is indicated by the return to normal red cell volume and the sustained reticulocytosis seen following thermal injury.

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A STUDY OF GAMMA GLOBULIN IN RHEUMATOID ARTHRITIS[1]

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For a number of years the attention of many investigators of rheumatoid arthritis has centered on the abnormality of the serum proteins in this disease, clinically manifested by a high globulin. Changes in the electrophoretically determined serum globulins have been reported (1), and to them the unusual agglutinating and precipitating properties of rheumatoid sera have been attributed (2–6). Recently evolved techniques for the isolation of plasma proteins by precipitation in cold alcohol (7, 8) and the current availability of radioisotopes for labelling purposes (9, 10) have provided another method for studying the fate of plasma proteins in blood and tissues.

A study of gamma globulin in patients with rheumatoid arthritis by means of these newer techniques is the subject of the present report.

1. Groups of patients with rheumatoid arthritis and groups of normal controls were given intravenous injections of I^{131}-tagged Fraction II (gamma globulin) and similarly tagged Fraction V (albumin). The rate of disappearance of these two proteins from the blood during the first two weeks was determined from the radioactivity remaining in serial plasma samples.

2. The *in vitro* adsorption by defatted joint tissue stromata of I^{131}-tagged samples of rheumatoid and control plasma Fraction II was studied.

3. Tagged Fractions II and V were given intravenously in different experiments to rheumatoid and non-rheumatoid subjects just before orthopedic operations. The uptake of radioactivity was measured in specimens of joint tissues removed during the operative procedure.

MATERIALS AND METHODS

Plasma fractionation. Plasma fractions were prepared by a modification (7) of Cohn's cold alcohol precipitation technique (Method No. 10) (8). The fractionation was further modified in this laboratory to facilitate radioactive tagging of the isolated proteins. The collection of fractions on glass filters by means of suction was replaced by sedimentation of precipitates in a refrigerated centrifuge; in the latter part of the fractionation procedure protein pastes were taken up in 0.04 M instead of 0.15 M saline, so that the anion exchange resin used subsequently to remove excess I^{131} and its carrier would not become saturated with chloride. Fraction II, consisting primarily of gamma globulin, was prepared from plasma pools derived from normal individuals as well as from separate pools derived from patients with rheumatoid arthritis. Fraction V, albumin, was obtained from a commercial source.[3]

Iodination. Iodination was accomplished by equilibrating the protein solutions against I^{131} in a carrier of iodine and potassium iodide. The technique was a modification of the methods used by Latta (9) and by Pressman (10). Since the iodine is taken up by the tyrosine molecules in protein the first step in determining the amount of carrier to be used was a Folin-Ciocalteu tyrosine analysis (11) of the protein solution. The assumption was then made that for gamma globulin 60 moles of tyrosine were contained in one mole of protein and for albumin 20 moles of tyrosine per mole of protein (12). Preliminary experiments revealed that satisfactory iodination could be accomplished by using a ratio of 6 moles of I_2 to one mole of protein. Uptake of radioactivity by the protein when these proportions were used varied from 20 to 40 per cent. Thus, since the carrier solution contained 5×10^{-6} mg. of I_2 per ml., the amount of carrier to be used could be calculated. One millicurie of I^{131} was added to the appropriate volume of carrier and this, following adjustment of pH to 8.5 – 9.0 by means of a glycine buffer, was added to the protein solution. Uncombined iodine was removed by dialysis against a glycine-buffered (pH 8.5 – 9.0) 0.04 M saline solution containing an anion exchange resin IRA-400.[4] At the end of the dialysis unbound radioactivity in the protein solution was found to be less than 1 per cent.

Determination of radioactivity. Counts of radioactivity were made with an end-window Geiger-Muller tube and a Tracerlab autoscaler. Duplicate ml. samples were pipetted into planchettes and allowed to air dry. Cracking occurred occasionally but was not sufficiently common to prove a hindrance. No correction was made for absorption, since in each section of the work pro-

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[4] Rohm and Haas Co., Philadelphia, Pennsylvania.

tein concentrations were approximately the same and this value was considered to be constant.

Adsorption of isoagglutinins. Traces of isoagglutinins were adsorbed from pooled Fraction II by three successive equilibrations at room temperature of given volumes of protein solution with equal volumes of thoroughly washed (eight times) human type AB, Rh positive, erythrocytes.

Preparation of tissue for in vitro adsorption studies. Synovial and adjacent joint capsule biopsies[5] obtained during the course of surgical procedures were placed in stoppered containers, frozen by immersing the containers in a mixture of solid CO_2 and 95 per cent ethanol, and stored at $-20°C$. Stromata were prepared by thawing the material, mincing it in saline at 0 to 4°C., grinding it in a tissue grinder,[6] washing the fragments by centrifugation from saline, and lyophilizing the sediment. The dried material was pulverized and extracted twice with cold ether. The resulting removal of fat provided a tissue preparation composed of evenly divided particles which could be readily suspended in saline.

Measurement of in vitro tissue uptake of globulin. For uptake studies 1.5 ml. aliquots of the iodinated plasma fractions were mixed with equal volumes of 2 per cent suspensions of the tissue particles, incubated for 40 to 60 minutes at 37°C., and washed by centrifugation from saline. The tissues were resuspended in 3 ml. volumes. Counts were made on duplicate 1 ml. aliquots of these tissue suspensions, on the supernates of the last washings, and on aliquots of the original tagged fraction. The radioactivity in the last washings were in all instances at, or nearly at, background.

Measurement of in vivo tissue uptake of globulin. Biopsy specimens from patients who had received a tagged plasma fraction preceding surgery were washed free of blood with saline before further study. In the initial experiments radioactivity was expressed as counts per gram of tissue before and after desiccation. In subsequent experiments radioactivity was expressed as counts per 10 mg. of tissue nitrogen as well. Samples were weighed wet and then digested in a mixture of concentrated nitric acid, 30 per cent hydrogen peroxide and silver nitrate in a water bath at 80°C. (13). In this process the iodine was precipitated by silver nitrate and the precipitate was collected by filtration. The section of filter paper holding the precipitate was placed in a planchette and counted in the usual manner. Nitrogen assays on the filtrate were determined with Nessler's solution. The results were recorded as counts per 10 mg. of nitrogen. This value was compared with the number of counts per ml. of plasma obtained at the same time that the biopsy was taken. It was assumed that the plasma total nitrogen did not vary significantly from the value of 10 mg. per ml.

[5] We are indebted to Doctors John G. Kuhns, Theodore A. Potter, Robert S. Hormell, George Van Gorder, Thomas B. Quigley, Henry Banks, and Joseph Fleming, whose interest and cooperation made possible the procurement of the tissue specimens.

[6] Ten Broeck grinder. Scientific Glass Apparatus Co., Bloomfield, New Jersey.

RESULTS

Part I. Disappearance of tagged fractions

The disappearance of intravenously injected Fraction II was studied in eight experiments. In four of these, gamma globulin was obtained from pooled plasma samples of normal individuals; in the other four the gamma globulin came from patients with active rheumatoid arthritis. Healthy young hospital personnel were used as normal subjects; the rheumatoid arthritis patients were somewhat older and were all hospitalized with moderate to severe disease. From six to ten individuals were represented in each plasma pool studied. In each experiment there were eight recipients: Four patients with rheumatoid arthritis and four normal controls. Recipients were given Lugol's solution daily to prevent localization of I^{131} in the thyroid.

The data describing the disappearance of radioactivity from the circulating blood are presented in Figures 1, 2, and 3. A semi-logarithmic plot of the plasma count, expressed as per cent of initial (generally thirty-minute) count, was made against time. It is evident in Experiments II, III, IV, and V (Figure 1) that there was a more rapid loss of the radioactive material from the blood of the rheumatoid arthritis patients than there was from that of the normal persons. Neither patients nor control recipients, however, differentiated between normal and rheumatoid plasma protein fractions. In Experiments II, IV, and V, the average tagged-protein half-life in the normal persons was found to be 7.7, 8.3, and 7.2 days, respectively, while the same averages for the rheumatoid arthritis group appeared to be one to two days shorter. The values obtained on the fourth and eleventh post-injection days in Experiments II through V, when subjected to statistical analysis, gave a probability value of 0.01. It was difficult to form a valid estimate of the half-life of the materials injected into the rheumatoid subjects, as plots of the data did not form straight lines after the second day.

In Experiment III the disappearance of radioactive material in both patients and controls was more rapid than in the previous experiments.

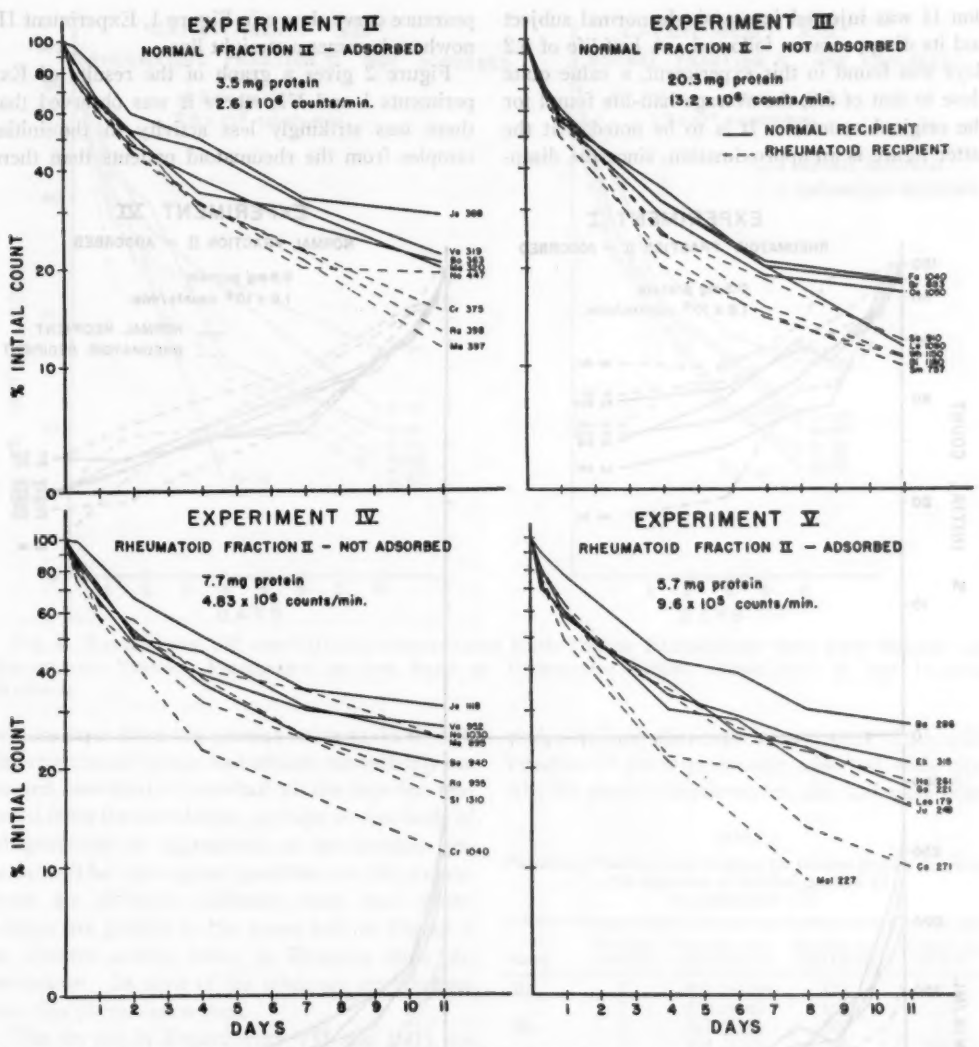

FIG. 1. EXPERIMENTS II–V, ILLUSTRATING THE MORE RAPID DISAPPEARANCE OF RADIOACTIVE MATERIAL FROM THE BLOOD OF RHEUMATOID INDIVIDUALS AS COMPARED TO NORMALS

The numbers accompanying each line refer to the counts per minute per milliliter of the initial (30-minute) plasma sample.

Gamma globulin used in this experiment was provided by Dr. J. L. Oncley of the Department of Physical Chemistry at Harvard Medical School; in the other experiments the Fraction II was prepared by the authors as described above. The test subjects of Experiment III were injected with aliquots of the material which had been tagged in the usual manner. The remainder was dialyzed against a Veronal buffer at $\mu = 0.1$ M and pH 8.6 in preparation for re-examination by electrophoresis. During dialysis an insoluble precipitate formed. Electrophoretic analysis of the protein remaining in solution indicated that it was unchanged gamma globulin. This supernate contained approximately half the original radioactivity. An aliquot of this soluble dialyzed Frac-

tion II was injected into a single normal subject and its disappearance followed. A half-life of 6.2 days was found in this experiment, a value quite close to that of 6.0, the average half-life found for the original material. It is to be noted that the latter figure is an approximation, since the disappearance curve shown in Figure 1, Experiment II, nowhere becomes a straight line.

Figure 2 gives a graph of the results of Experiments I and VI, where it was observed that there was strikingly less activity in the initial samples from the rheumatoid patients than there

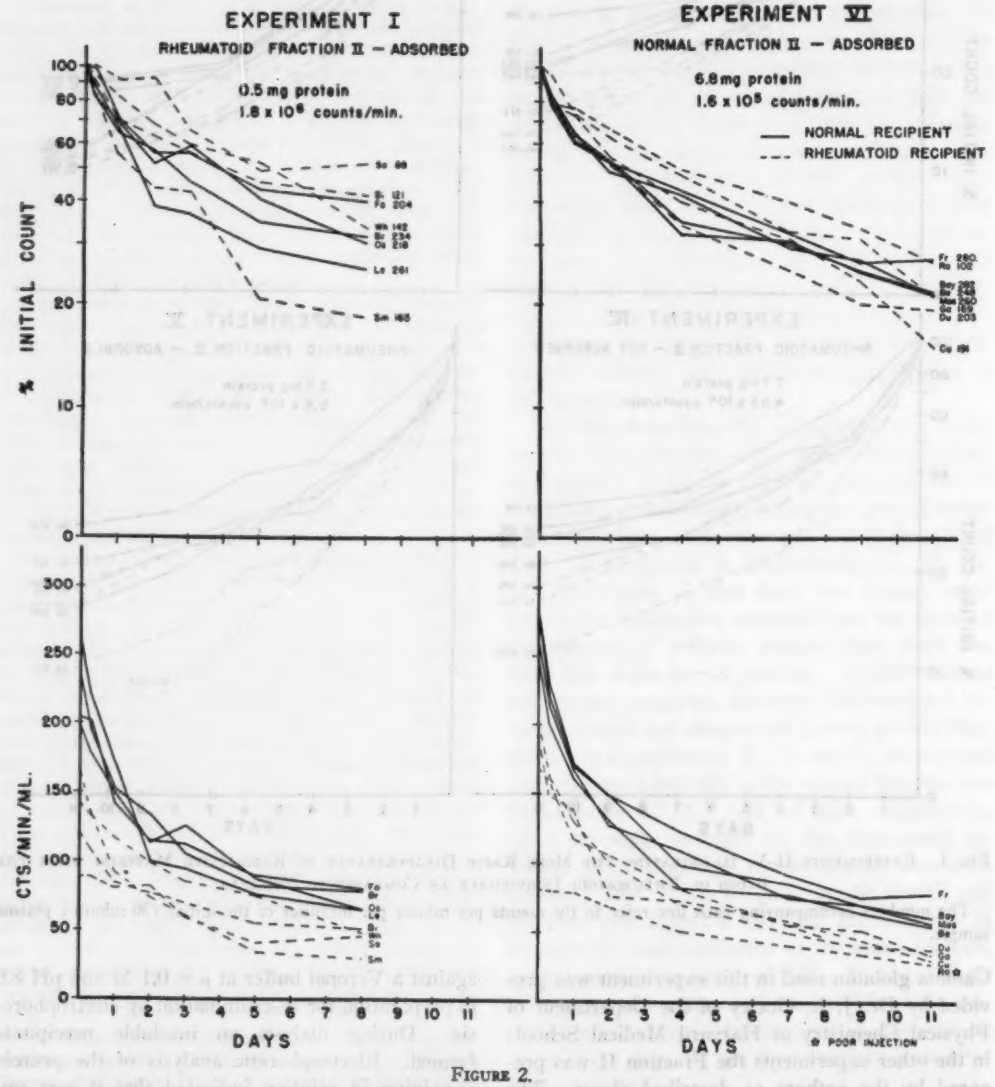

FIGURE 2.

In Experiments I and VI there was an immediate removal of significant amounts of radioactive material from the blood of rheumatoid individuals, after which the curves were almost identical. The upper curves are re-plotted below against counts per minute per milliliter better to illustrate the significance of the lack of overlap in the initial (30-minute) plasma samples.

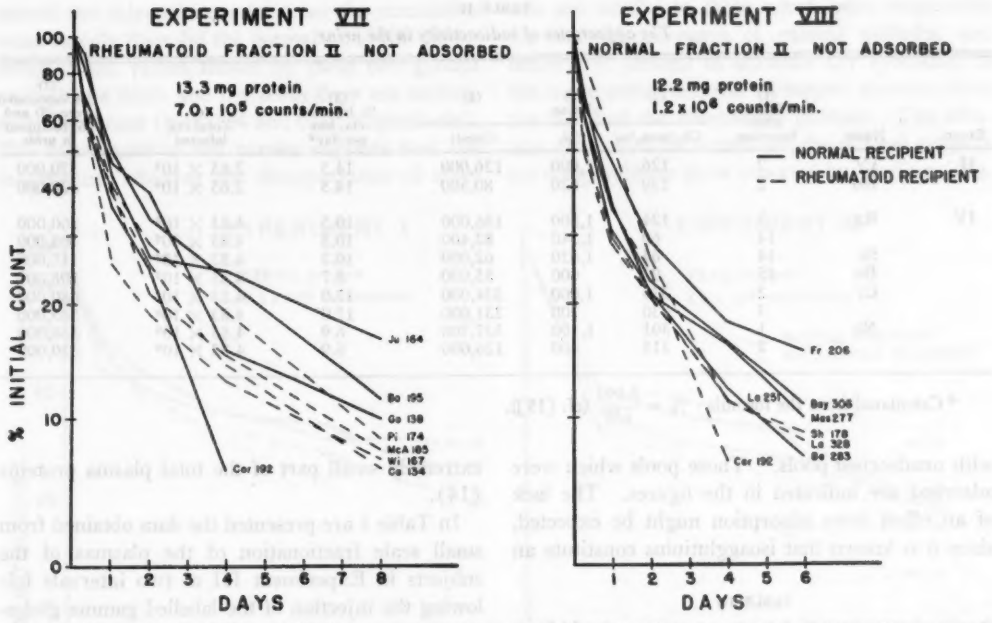

FIG. 3. EXPERIMENTS VII AND VIII, ILLUSTRATING THE RAPID LOSS OF RADIOACTIVITY FROM BOTH NORMAL AND RHEUMATOID PERSONS, PRESUMABLY ON THE BASIS OF DEGRADATION DURING PREPARATION OF THE INJECTED MATERIAL

was in those from the normal subjects, as though the rheumatoid group had almost immediately removed one-third to one-half of the injected material from the circulation, perhaps on the basis of phagocytosis of aggregated or precipitated material. The subsequent portions of the curves were not definitely different from each other. Values are plotted in the lower half of Figure 2 as absolute counts, better to illustrate these observations. In none of the other six experiments was this phenomenon seen.

The results in Experiments VII and VIII are presented in Figure 3. In each of these instances there was unusually rapid disappearance of radioactivity from the blood, not only of the patients but of the controls as well, suggesting that during the isolation and iodination procedures in these experiments there was serious degradation of the protein. Again, this phenomenon was more striking in rheumatoid subjects than in controls, a finding in keeping with the tendency noted earlier, in Experiments II–V.

To rule out the possible influence of the known isoagglutinogens as complicating factors in this study, half of the experiments were done with Fraction II pools previously adsorbed with type AB, Rh positive erythrocytes, and half were done

TABLE I

Partition of radioactivity between the plasma protein fractions after injection of labelled fraction II in experiment III

Name	Day after injection	Cts./min./ml. unfractionated	Cts./min./ml. I & II & III	Ct./min./ml. IV & V
Br	2	421 (177)*	172	7.1
	7	176 (106)	109	3.0
Ra	2	503 (211)	202	5.2
	7	221 (130)	124	10.3
Le	2	512 (215)	185	8.9
	7	212 (125)	83	0.8
Os	2	469 (197)	232	11.8
	7	202 (119)	116	5.3
Bi	2	437 (183)	210	2.0
	7	173 (102)	81	7.2
Sa	2	411 (172)	172	3.0
	7	164 (97)	80	7.5
Sm	2	319 (134)	118	7.1
	7	110 (65)	57	6.3
Wh	2	513 (215)	184	4.0
	7	188 (109)	111	12.7

* Figures in parentheses represent the corrections for the decay that occurred in the ten-day interval between the readings of the unfractionated and fractionated materials. These corrected values are to be compared with the readings obtained on the fractionated material.

TABLE II

The appearance of radioactivity in the urine

Exper.	Name	(1) Days after injection	(2) Urine Cts./min./ml.	(3) Urine Vol.	(4) Urine Counts	(5) % Injected cts. lost per day*	(6) Total cts. injected	(7) Cts. calculated from (5) and (6) to appear in urine
II	Cr	2	126	1,000	126,000	14.5	2.65×10^8	320,000
	No	2	139	620	80,500	14.5	2.65×10^8	320,000
IV	Ra	7	124	1,500	186,000	10.5	4.83×10^8	260,000
		14	60	1,340	82,400	10.5	4.83×10^8	108,000
	St	14	64	1,020	62,000	10.2	4.83×10^8	110,000
	Bo	15	92	600	55,000	8.7	4.83×10^8	108,000
	Cr	2	334	1,000	334,000	17.0	4.83×10^8	680,000
		3	330	700	231,000	17.0	4.83×10^8	563,000
	No	1	301	1,100	537,700	6.9	4.83×10^8	334,000
		2	315	400	126,000	6.9	4.83×10^8	310,000

* Calculated from the formula: $\% = \frac{0.693}{t/2}$ (cf. [15]).

with unadsorbed pools.† Those pools which were adsorbed are indicated in the figures. The lack of an effect from adsorption might be expected, since it is known that isoagglutinins constitute an extremely small part of the total plasma proteins (14).

TABLE III

Results of scanning with beta gamma survey meter 24 hours after injection in experiment V

	cts./min.		cts./min.
Ha		Mal	
Background	90	Background	90
Thyroid	180	Thyroid	120
Bladder	120	Bladder	140
Left knee	110*	Right hand	140*
Kidney	120	Right kidney	120
Liver	120	Left kidney	100
Left wrist	100*	Right knee	120*
	cts./min.		cts./min.
Co		Leo	
Background	90	Background	90
Thyroid	140	Right hand	100
Right hand	100	Liver	120
Liver	140	Bladder	100
Bladder	120	Right kidney	90
Right kidney	100	Left kidney	90
Left kidney	120	Right knee	90
Right knee	100*		
Left knee	100*		

	cts./min.
Go	
Background	100
Thyroid	120
Left hand	110
Liver	120
Bladder	130
Right kidney	110
Left kidney	110

* Joints involved by rheumatoid arthritis.

† The isoagglutinins are concentrated in Fraction III but by qualitative testing significant traces of these substances can also be found in Fraction II.

In Table I are presented the data obtained from small scale fractionation of the plasmas of the subjects in Experiment III at two intervals following the injection of the labelled gamma globulin. It is evident that the injected material remained associated with Fraction I plus II plus III, no significant amount appearing in Fraction IV plus V. Similar results were obtained in several of the other experiments, the longest post-injection interval studied being two weeks.

The available data on urinary excretion of radioactivity are shown in Table II. In only one instance (Subject "No," first day of Experiment IV) was as much radioactivity found in the urine as might have been expected from a consideration of the plasma decay curves. This is illustrated by a comparison of columns No. 4 and No. 7, which indicates that approximately half the expected count was found in the urine. The amounts of iodine lost in the feces and perspiration were not measured but one would not have expected them to be high.

The surface of the body was scanned, as illustrated in Table III. There was no evidence for any specific localization of radioactivity over joints afflicted by arthritis.

In Figure 4 are shown the disappearance curves for commercial human albumin tagged with I^{131} following intravenous administration in patients and controls. As with globulin, the patients re-

moved the injected material from the circulation more rapidly than did the normals, and the differences in the values shown by these two groups on both the ninth and the tenth days are statistically significant ($p = 0.04$ and 0.01, respectively). The appearance of the curves suggests that the mechanisms effecting the disappearance of albumin are similar to those which were responsible for the disappearance of gamma globulin, and hence it is difficult to attribute any specificity to the more rapid removal of tagged globulin from the blood of the rheumatoid patients. The albumin disappearance curves for the normal subjects are comparable to those reported by Sterling (15).

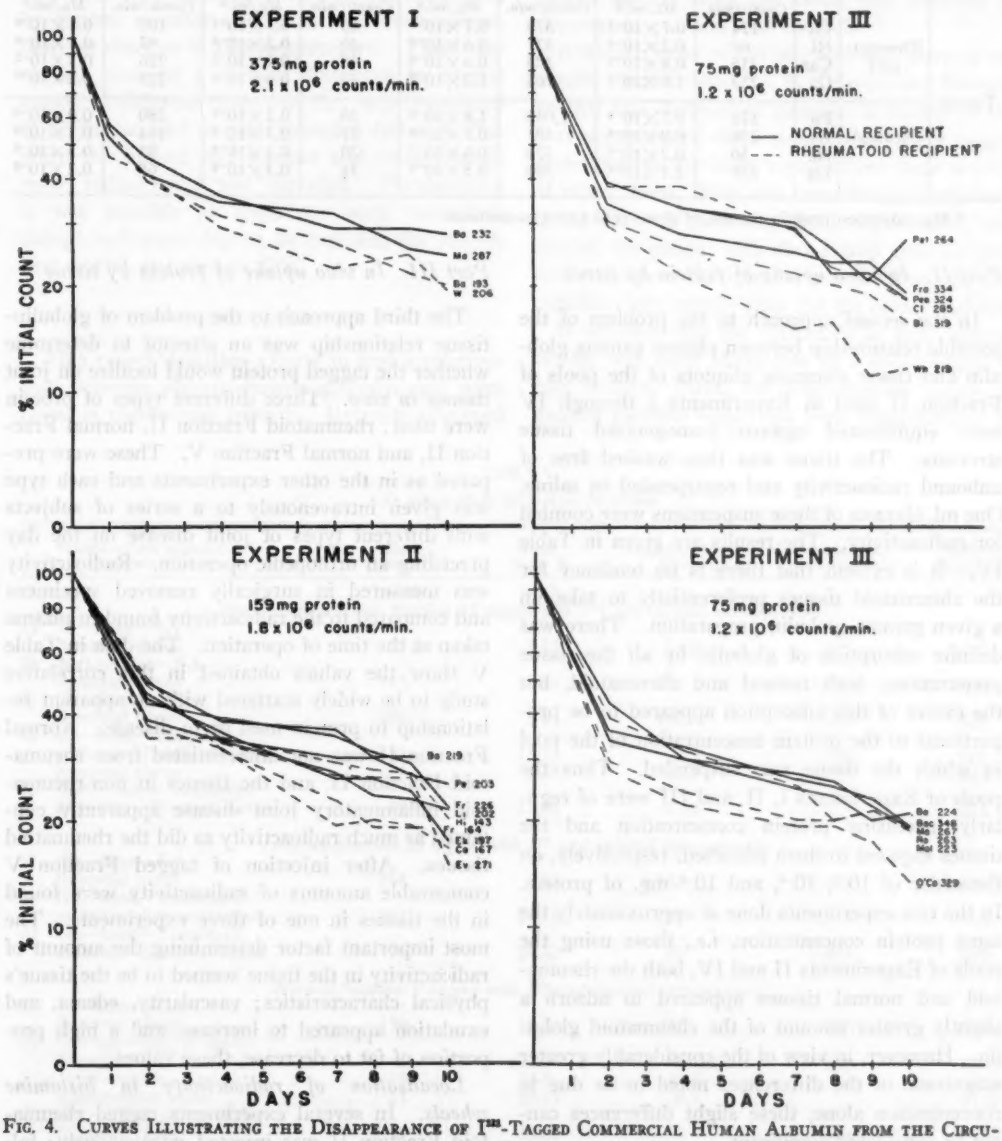

FIG. 4. CURVES ILLUSTRATING THE DISAPPEARANCE OF I^{131}-TAGGED COMMERCIAL HUMAN ALBUMIN FROM THE CIRCULATION OF NORMAL INDIVIDUALS AND OF PATIENTS WITH RHEUMATOID ARTHRITIS

TABLE IV

In vitro adsorption of fraction II by tissue

Data on protein			Rheumatoid fr. II pool of exp. I 0.017 mg. protein/ml. 4.44 ×10⁵ cts./ml. 2.67 ×10⁵ cts./mg. protein		Rheumatoid fr. II pool of exp. IV 0.074 mg. protein/ml. 4.28 ×10⁵ cts./ml. 5.7 ×10⁵ cts./mg. protein		Normal fr. II pool of exp. II 0.085 mg. protein/ml. 1.05 ×10⁵ cts./ml. 1.23 ×10⁵ cts./mg. protein		Normal fr. II pool of exp. III 0.290 mg. protein/ml. 9.08 ×10⁵ cts./ml. 3 ×10⁵ cts./mg. protein	
			Counts/min.	*Mg./ml.**	*Counts/min.*	*Mg./ml.**	*Counts/min.*	*Mg./ml.**	*Counts/min.*	*Mg./ml.**
Tissues	Rheumatoid	Ca	174	0.7×10^{-4}	379	0.7×10^{-3}	40	0.3×10^{-3}	107	0.4×10^{-2}
		Hi	60	0.2×10^{-4}	321	0.6×10^{-3}	30	0.2×10^{-3}	97	0.3×10^{-2}
		Car	215	0.8×10^{-4}	323	0.6×10^{-3}	48	0.4×10^{-3}	226	0.8×10^{-2}
		Ga	257	1.0×10^{-4}	765	1.3×10^{-3}	70	0.6×10^{-3}	225	0.8×10^{-2}
	Normal	Fe	176	0.7×10^{-4}	1,040	1.8×10^{-3}	26	0.2×10^{-3}	280	0.9×10^{-2}
		Br	236	0.9×10^{-4}	381	0.7×10^{-3}	37	0.3×10^{-3}	164	0.5×10^{-2}
		He	50	0.2×10^{-4}	370	0.6×10^{-3}	20	0.2×10^{-3}	85	0.3×10^{-2}
		Da	559	2.1×10^{-4}	306	0.5×10^{-3}	34	0.3×10^{-3}	68	0.2×10^{-2}

* Mg. adsorbed protein per ml. of 2 per cent tissue suspension.

Part II. In vitro uptake of protein by tissue

In the second approach to the problem of the possible relationship between plasma gamma globulin and tissue elements, aliquots of the pools of Fraction II used in Experiments I through IV were equilibrated against homogenized tissue stromata. The tissue was then washed free of unbound radioactivity and resuspended in saline. One ml. aliquots of these suspensions were counted for radioactivity. The results are given in Table IV. It is evident that there is no tendency for the rheumatoid tissues preferentially to take up a given gamma globulin preparation. There was definite adsorption of globulin by all the tissue preparations, both normal and rheumatoid, but the extent of this adsorption appeared to be proportional to the protein concentration of the pool in which the tissue was suspended. Thus the pools of Experiments I, II, and III were of regularly increasing protein concentration and the tissues exposed to them adsorbed, respectively, on the order of 10^{-4}, 10^{-3}, and 10^{-2} mg. of protein. In the two experiments done at approximately the same protein concentration, *i.e.*, those using the pools of Experiments II and IV, both the rheumatoid and normal tissues appeared to adsorb a slightly greater amount of the rheumatoid globulin. However, in view of the considerably greater magnitude of the differences noted to be due to concentration alone, these slight differences cannot be considered significant.

Part III. In vivo uptake of protein by tissue

The third approach to the problem of globulin-tissue relationship was an attempt to determine whether the tagged protein would localize on joint tissues *in vivo*. Three different types of protein were used: rheumatoid Fraction II, normal Fraction II, and normal Fraction V. These were prepared as in the other experiments and each type was given intravenously to a series of subjects with different types of joint disease on the day preceding an orthopedic operation. Radioactivity was measured in surgically removed specimens and compared to the radioactivity found in plasma taken at the time of operation. The data in Table V show the values obtained in this correlative study to be widely scattered with no apparent relationship to protein used or to disease. Normal Fraction II was not differentiated from rheumatoid Fraction II, and the tissues in non-rheumatoid inflammatory joint disease apparently contained as much radioactivity as did the rheumatoid tissues. After injection of tagged Fraction V comparable amounts of radioactivity were found in the tissues in one of three experiments. The most important factor determining the amount of radioactivity in the tissue seemed to be the tissue's physical characteristics; vascularity, edema, and exudation appeared to increase, and a high proportion of fat to decrease, these values.

Localization of radioactivity in histamine wheals. In several experiments tagged rheumatoid Fraction II was injected intravenously; fol-

lowing this, histamine wheals were produced in the skin (by injection of 0.1 ml. of histamine phosphate 1:1000 intradermally) and the appearance of radioactivity in these wheals measured. In general the number of counts correlated fairly well with the size of the histamine wheal and flare. However, to a measurable extent, the amount of radioactivity over an area of skin was influenced by general vascular effects, such as those accompanying changes in skin temperature. There was on one occasion a marked difference in control counts at different times and this was considered to be due, at least in part, to such a factor, in that when the subject was warmer and more relaxed more radioactivity was recorded. Furthermore it was possible to reproduce such variations, though to a lesser degree, by exposing the subject first to 4°C. and then to 37°C.

DISCUSSION

This report describes efforts to find a relationship between plasma gamma globulin and joint tissue in rheumatoid arthritis. Recently advanced techniques of plasma fractionation and radio isotope tagging were employed. I^{131}-labeled Fraction II (gamma globulin) of both normal and rheumatoid origins was equilibrated *in vitro* and *in vivo* with normal and rheumatoid joint tissue and the uptake of radioactivity by the tissue measured. In addition, the disappearance of similarly tagged protein from the blood was followed in rheumatoid patients and controls.

Although none of the subjects and none of the tissues studied appeared to differentiate between normal and rheumatoid globulin, a review of the curves expressing the disappearance of the tagged proteins from the blood revealed two facts worthy of note. First, there was considerable variability in the different gamma globulin experiments, and second, the patient with rheumatoid arthritis generally removed the injected protein from the circulation more rapidly than did the normal subject.

It was felt that the variability in experimental results was most likely due to a combination of two factors: First, the heterologous nature of gamma globulin which has been demonstrated by

TABLE V

In vivo uptake of protein by tissue

Patient—Joint operated—Diagnosis—Tissue	Total tissue	Total tissue N	Cts./min./10 mg. tissue N	Cts./min./ml. Plasma	Tissue cts. / Plasma cts.
	Cts./min.	*(mg.)*			
Rheumatoid arthritis fraction II tagged with I^{131} and injected intravenously					
1) Sc—knee—RA—Synovial tissue	12.4	5.75	21.6	504	0.042
2) McL—knee—RA—Synovial tissue	198	13.17	150	1,110	.136
3) Ty—knee—RA—Synovial tissue	388	3.9	995	1,205	.825
4) Le—knee—Traumatic arthritis—Patellar fat pad	2	13.73	2	970	.001
Synovial tissue	13	1.58	82.3		.085
5) McC—knee—Ruptured meniscus—Synovial tissue	18	17.14	10.5	1,015	.014
Normal fraction II tagged with I^{131} and injected intravenously					
1) Ha—knee—RA—Synovial tissue	764	4.68	1,632	1,120	1.46
2) Ne—knee—Traumatic arthritis—Synovial tissue	802	29.76	269	1,372	.196
3) Ga—knee—Traumatic arthritis—Synovial tissue	79	20.52	38	1,861	.020
Normal fraction V tagged with I^{131} and injected intravenously					
1) Fl—knee—Ra—Synovial tissue	130	16.8	77	290	.266
2) Gr—hip—deg. jt. dis.—Muscle	4	127.0	.315	497	.0006
Joint capsule	25	23.8	10.5		.0212
Ligamentum teres	14	23.75	6.1		.0122
3) Gi—M.T.P. joints—RA—Synovial tissue	4	38.36	1	306	

others on the basis of immunochemical (16) and solubility (17) criteria; and second, unrecognized variations in the procedures by which the proteins were prepared. It is possible that varying degrees of denaturation may have occurred which would make the injected material more or less liable to removal from the circulation by the processes available to the body for dealing with foreign proteins.[8]

The more rapid disappearance of injected protein from the circulations of the rheumatoid patients may have been due to one or more of several causes. It is possible that this was only a part of a general difference in protein metabolism such as that responsible for the negative nitrogen balance that is often found in this disease (18). The effect of the age of the individual on the half-life of gamma globulin as described by Dixon, Talmage, Maurer, and Deichmiller (19) was also considered since in the present study the patients were generally older than the controls; however, no correlation between the rates of disappearance and the subjects' ages could be detected. The mere existence of chronic disease may have affected the curves, and future studies on patients with other conditions may serve to shed some light on the specificity of this phenomenon. The possibility was considered that the low values in the rheumatoid patients could have been a distributional effect dependent upon a gradual equilibration of the injected material through abnormally large total body protein pools, the latter, perhaps, in part induced by a tendency for proteins to leak into areas of inflammation. This possibility could not be excluded, but to evoke it as an explanation would require the added hypothesis that in the present study the time needed for the proteins to reach this equilibrium was considerably longer than the one to three-day period that has been described by others in similar studies (15). Finally, this observation can be interpreted as indicating that the patient with rheumatoid arthritis possesses immunological or other mechanisms whereby it is possible for him to remove aggregated or precipitated protein material from the blood stream more rapidly than is possible for the normal control.

[8] Since the preparation of this paper an article has appeared by Berson, S. A., Yalow, R. S., Schreiber, S. S., and Post, J., Tracer experiments with I-131-labeled human serum albumin: distribution and degradation studies. J. Clin. Invest., 1953, **32**, 746. These authors call attention to the variability which may be found in presumably homogeneous proteins which are labeled when they are studied by measuring their disappearance from the blood stream after intravenous injection. They emphasize the importance of viewing with skepticism the concept that such disappearance rates accurately reflect the actual metabolism of the protein under investigation, as the material injected may have lost a significant portion of its original identity.

SUMMARY

No evidence was obtained to support the concept of a specific relationship between joint tissue and plasma gamma globulin in rheumatoid arthritis. Neither normal nor rheumatoid subjects, nor their independently studied joint tissues *in vitro* or *in vivo*, differentiated between normal and rheumatoid I^{131}-labeled Fraction II.

It was observed that the elimination of injected proteins from the circulation in rheumatoid patients was more rapid than in normal subjects.

The possible mechanisms responsible for this finding are discussed.

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EFFECTS OF SIMULTANEOUS OR PRIOR INFUSION OF SUGARS ON THE FATE OF INFUSED PROTEIN HYDROLYSATES [1, 2]

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Glucose usually is included in amino acid solutions for intravenous infusion. This has seemed desirable because the amino acids can spare body protein only when sufficient calories are provided. Although the calories so gained (usually 200 for a liter of hydrolysate) are far from enough for this purpose, they do add to the total and besides they might serve particularly efficiently being presented at the same time as the amino acids.

On the other hand Elman and his co-workers (1) have reported improved retention of the nitrogen of a partial hydrolysate when fructose replaced the usual glucose. In explanation these writers suggest that the cellular uptake of the glucose is relatively too late to spare the amino acids optimally, whereas the fructose is metabolized more quickly.

The present study was undertaken to compare the effects of glucose and fructose on the fate of the amino acids and peptides of infused hydrolysates. We have found that the presence of either sugar in certain commercial protein hydrolysates interferes with the tissue utilization of the peptides present; the effect increases in the order 10 per cent fructose, 5 per cent glucose, 10 per cent glucose. It is completely avoided if the sugars are added to the hydrolysate sterilely just before infusion or if the sugars are infused in advance of the hydrolysate. Therefore, we conclude that substances which interfere with peptide utilization are formed during autoclaving by interaction between the sugars and constituents of the hydrolysate.

Interference has been observed also with the utilization of the *free* amino acids especially at a 10 per cent glucose level. This interference was not due to autoclaving and therefore appeared to be due to the sugar itself acting upon the renal reabsorption of the amino acids.

If the advantage of fructose arises from its more rapid utilization, this advantage should also be attainable with either sugar by starting its infusion before the hydrolysate. In the course of seeking ways of avoiding the above interference, we have observed that the prior infusion of either sugar substantially diminishes the immediate augmentation of nitrogen excretion which otherwise occurs. Elman has reported, with unfavorable results, a single test of the reverse order of presentation, *i.e.*, amino acids in the forenoon and glucose in the afternoon (2).

EXPERIMENTAL

Commercially available hydrolysates were studied. The partial hydrolysate of fibrin (Aminosol® [3]) was supplied in four forms: a sugar-free preparation, one containing 5 per cent glucose, one containing 10 per cent glucose, and one containing 10 per cent fructose. The enzymatic partial hydrolysate of casein + pig pancreas (Amigen® [3]) was used as a sugar-free preparation, another with 5 per cent glucose, and a third with 10 per cent fructose. A partial hydrolysate of bovine plasma proteins (Travamin® [3]) was used as a sugar-free solution and one containing 5 per cent glucose. All preparations were indicated to be of 5 per cent strength, and analyzed by us for free and bound amino acids (Table I).

The sugar-free fibrin hydrolysate was supplemented in

[1] This investigation was supported in part by a grant (C-1268) from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service, and by a grant from the Abbott Laboratories, Inc.

[2] A preliminary report of this work has been made to The Fundamental Forum, American College of Surgeons, Atlantic City, November, 1954.

[3] The fibrin hydrolysate Aminosol® was kindly supplied by Dr. Douglas V. Frost of the Abbott Laboratories, in the several solutions listed. The casein-pig pancreas hydrolysate Amigen®, as well as fructose solutions, were supplied through the kindness of Dr. Warren Cox, Jr., Mead Johnson and Company. The plasma protein hydrolysate Travamin® is the product of the Baxter Laboratories, Inc., the samples having been obtained on the market.

TABLE I

Amino acids and peptides of hydrolysates studied—Manometric ninhydrin method at pH 2.5

Protein of origin	Sugar content	α-Amino acids	
		Free mM/L.	Bound mM/L.
Fibrin	None	224	130
Fibrin	10% fructose	198	117
Fibrin	5% glucose	203	149
Fibrin	10% glucose	202	113
Casein	None	235	129
Casein	10% fructose	251	115
Casein	5% glucose	249	131
Plasma proteins	None	173	161
Plasma proteins	5% glucose	175	170
Fibrin	10% glucose, freshly added	216.4	126.4
Fibrin	Same solution, autoclaved	217.5	126.0

some cases by adding to 1 liter of it 200 ml. of sterile 50 per cent glucose, under sterile precautions. This mixture was infused at a 20 per cent faster rate to compensate for its higher water content. When it was autoclaved, this was done at a pressure of 10 to 11 pounds per square inch for 10 minutes under a carbon dioxide atmosphere. A sugar-free double-strength solution of the hydrolysate of casein plus pancreas was fortified with sugar by adding to 500 ml. of it 200 ml. of 50 per cent glucose and 300 ml. of sterile water under the same conditions. These solutions were infused promptly after their preparation.

A uniform rate of infusion equivalent to 7.2 ml. per kilogram per hour of the 5 per cent protein hydrolysates was obtained by means of a flowmeter (Fischer and Porter Company "Triflat" variable-area flowmeter). This meter was calibrated for each of the solutions used. The above rate corresponds to an infusion time of a little over two hours for a liter of solution. The comparisons were made in each case with the same subject over an interval of a few days. Ten of the subjects were convalescent surgical patients who were estimated to have minimal metabolic disturbances; the other five were normal young persons. Blood was taken from an antecubital vein at the beginning, midpoint, just before the end, and 30 minutes after the end of the infusion. The subjects fasted from the evening before to three hours after the infusion. A pretest urine sample was collected from 10 P.M. (or 2 A.M.) to about 8 A.M.; the experimental urine was collected from the beginning of the infusion to three hours after its end. Collecting urine over an additional four hours was found only to intensify somewhat the differences in the wastage of free and bound amino acids reported here. The collection of urine was under the supervision of a member of the investigating staff and with the close cooperation of the subject.

The plasma and urine samples were analyzed for free and bound amino acids after removal of proteins (3, 4). Some simplification of the technique should make the determination of bound amino acids of plasma more attractive to other investigators. Accordingly the present procedure is described: Four aliquots of 6 ml. each of the picric acid filtrates were pipetted into four of the special Thunberg tubes described by Hamilton and Van Slyke (5). Two of these portions were analyzed immediately for amino acids by the carbon dioxide released by ninhydrin at pH 2.5 (5); 3 ml. of concentrated hydrochloric acid were added to each of the other two, and they were heated in a steam bath 24 hours. The stoppers were very nearly closed during the first 20 hours, and removed during the last few hours, until the volume had decreased to 0.5 to 1 ml. Evaporation was completed in a vacuum dessicator over potassium hydroxide pellets. Two ml. of water and a drop of bromcresol green were added, and the pH adjusted to a green color with 2 or 3 drops of N sodium hydroxide. Solid citrate buffer (6) was added in order to obtain a pH of 2.5, and the total amino acids were determined as usual. The urine (or in the presence of proteinuria, a dialysate of the urine) was subjected to similar analysis before (7) and after hydrolysis. A dialysis of the plasma filtrates before analysis would have made the exclusion of protein more rigorous (3), but changes during infusion of the small quantity of picric acid soluble protein were shown in the present instance to be insignificant, relative to the changes produced for plasma peptides.

The excretion rates for amino acids and peptides during and after the infusion were corrected for the comparatively small background (pretest) rates, to yield the extra excretion due to the infused material. The method of calculation has been illustrated earlier (4).

In part of the experiments the extra total urinary nitrogen excreted during and immediately after an infusion was estimated. In this case the infusions were separated by not less than 44 hours. The subject ate a supper of low and nearly constant protein content at 6 P.M. Abundant water was taken during the evening, at retiring, and upon being waked at 2 A.M., to promote a nearly maximal rate of urea elimination. A urine sample was collected from 2 A.M. for six to seven hours and the hourly rate of nitrogen excretion determined. The extra nitrogen above this background hourly rate excreted during the experimental period (infusion period + three subsequent hours) was then determined, and compared with the amount of nitrogen infused. Nitrogen excreted during a third period of four hours was also determined, but this quantity was not remarkably different for the various infused solutions. When a sugar was infused prior to the hydrolysate, the urine secreted during the sugar infusion was collected as a separate sample.

RESULTS AND DISCUSSION

Preliminary comparison of glucose and fructose

This study began as a comparison of the effects of glucose and fructose, each present in the hydrolysate at 10 per cent strength, on the fate of

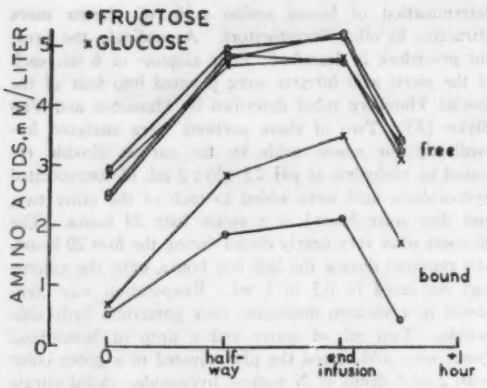

FIG. 1. PLASMA AMINO ACID AND PEPTIDE LEVELS DURING AND AFTER INFUSION OF FIBRIN HYDROLYSATES AUTOCLAVED WITH FRUCTOSE OR GLUCOSE (CONVALESCENT PATIENT)

The amino acid concentrations are in millimoles per liter. They may be converted to milligrams per cent of amino acid nitrogen by multiplying by 1.4.

the amino acids of the fibrin hydrolysate. These solutions had been autoclaved by the manufacturer with the sugar present. The two preparations were infused on alternate days for four days. The plasma amino acid tolerance curves were found to be very closely reproducible in a given subject, although rather different in different subjects. The curve always lay slightly higher in the presence of fructose than in the presence of glucose (Figure 1; also Figure 3, below). This was the opposite of the effect reported by Elman and his co-workers (1). Accordingly, the same comparison was made with the casein hydrolysate used by these workers; in every one of eight comparisons with either preparation the higher plasma amino acid levels were obtained with fructose, whether the glucose was at a 5 or 10 per cent concentration.

The urine analyses showed a striking difference, the loss of amino acids and peptides being about twice as great in the presence of glucose. Our first thought was that the larger diuresis usually obtained with the glucose-containing hydrolysate might account for this finding; in agreement with others we have found much higher glucose levels in the blood during the infusions than for fructose, and much larger losses of glucose into the urine. Therefore, comparable degrees of diuresis were induced by giving the subject water to drink during the fructose hydrolysate infusions. Figure 2 shows that the amino acid and peptide losses were unrelated to urine volume.

Much higher levels of peptides (that is, of nonprotein, bound amino acids) in the plasma during the infusions containing glucose showed that the greater peptiduria was consequent to a higher peptidemia and, therefore, prerenal in origin (illustrated in Figures 1 and 3).

General study of the effect of sugars in hydrolysates

Subsequent trial with sugar-free hydrolysates showed amino acid and peptide losses even lower than for the fructose-containing preparation; hence, the advantage of fructose over glucose is really only *a smaller disadvantage,* that is, a smaller interference in the presence of this sugar with amino acid and peptide utilization. The study was, therefore, extended to include fibrin hydrolysate solutions containing a) no sugar, b) 10 per cent fructose, c) 5 per cent glucose, and d) 10 per cent glucose. Figures 3 and 4 show

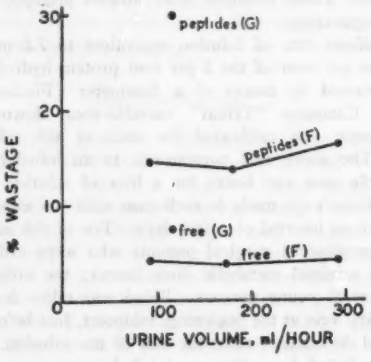

FIG. 2. LACK OF RELATION BETWEEN THE LOSSES OF PEPTIDES AND AMINO ACIDS AND THE URINE VOLUME (CONVALESCENT PATIENT)

(G) indicates the results with a fibrin hydrolysate containing 10 per cent glucose, autoclaved by the Abbott Laboratories after mixing; (F) gives the results with a parallel hydrolysate containing 10 per cent fructose, likewise autoclaved. The subject drank 750 and 1500 ml. of water during the last two fructose-containing infusions to produce the diuresis. In this and the succeeding figures the urinary wastage is shown as the per cent of the amount infused of that particular category (free or bound) of amino acid.

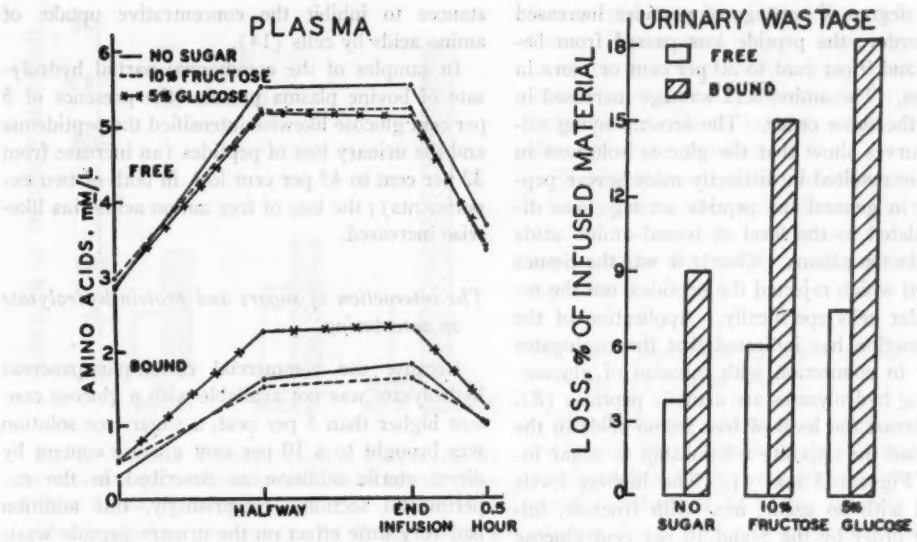

FIG. 3. PLASMA LEVELS AND URINARY WASTAGES OF FREE AND BOUND AMINO ACIDS WITH FIBRIN HYDROLYSATES, AS AFFECTED BY THE PRESENCE OF FRUCTOSE OR GLUCOSE (NORMAL SUBJECT)

The preparations had been autoclaved by the manufacturer after addition of the sugar.

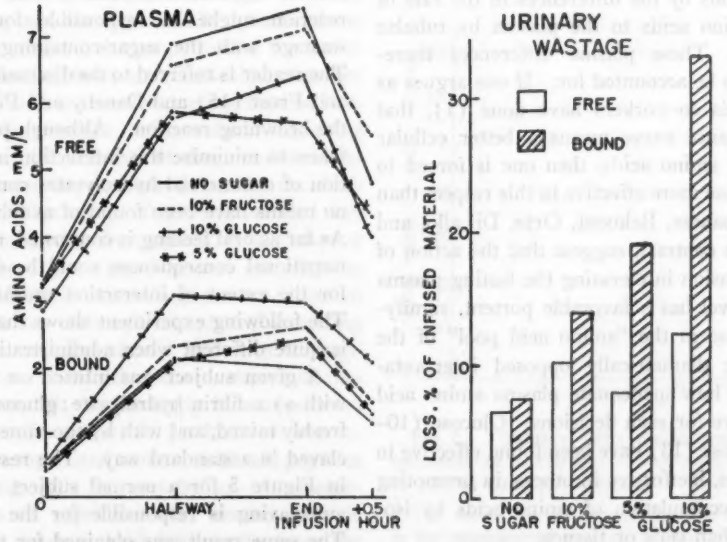

FIG. 4. PLASMA LEVELS AND URINARY WASTAGES OF FREE AND BOUND AMINO ACIDS WITH FIBRIN HYDROLYSATES, AS AFFECTED BY THE PRESENCE OF FRUCTOSE OR GLUCOSE (NORMAL SUBJECT)

The preparations had been autoclaved by the manufacturer after addition of the sugar.

that the degree of wastage of peptides increased in that order; the peptide loss passed from between 6 and 9 per cent to 30 per cent or more in this series. The amino acid wastage increased in roughly the same order. The accompanying tolerance curves show that the glucose solutions in every case resulted in distinctly more severe peptidemia; in general the peptide wastage was directly related to the level of bound amino acids reached in the plasma. Clearly it was the tissues in general which rejected the peptides, not the renal tubular cells specifically. Application of the biuret reaction has indicated that the conjugates excreted in connection with infusion of glucose-containing hydrolysates are actually peptides (8).

In contrast the level of free amino acids in the plasma had the opposite relationship to sugar infusion (Figures 3 and 4). The highest levels occurred with no sugar, next with fructose, followed in order by the 5 and 10 per cent glucose preparations. The increased loss of free amino acids with elevated blood sugar levels, therefore, appears in contrast to have mainly a renal origin.

Simple calculations show that one cannot account for the differences in the plasma levels of free amino acids by the differences in the rate of return of amino acids to the plasma by tubular reabsorption. These plasma differences therefore remain to be accounted for. If one argues as Elman and his co-workers have done (1), that a lower tolerance curve means a better cellular uptake of the amino acids, then one is forced to consider glucose more effective in this respect than fructose. Albanese, Belmont, Orto, DiLallo, and Rossy (9), in contrast, suggest that the action of a fructose infusion in elevating the fasting plasma amino acid level has a favorable portent, signifying an increase in the "amino acid pool" of the body. These diametrically opposed interpretations indicate how inadequate plasma amino acid levels alone are for such decisions. Glucose (10–12) and fructose (13) have been found effective in some instances, ineffective in others, in promoting the cellular accumulation of amino acids by isolated mammalian cells or tissues.

The results do not permit one to exclude the elevated plasma peptides as a factor in the depressed renal reabsorption of the amino acids. Peptides have been shown at least in some instances to inhibit the concentrative uptake of amino acids by cells (14).

In samples of the commercial partial hydrolysate of bovine plasma proteins the presence of 5 per cent glucose likewise intensified the peptidemia and the urinary loss of peptides (an increase from 32 per cent to 42 per cent lost, in both of two experiments); the loss of free amino acids was likewise increased.

The interaction of sugars and protein-hydrolysate on autoclaving

Because the commercial casein-plus-pancreas hydrolysate was not available with a glucose content higher than 5 per cent, a sugar-free solution was brought to a 10 per cent glucose content by direct sterile addition as described in the experimental section. Surprisingly, this addition had very little effect on the urinary peptide wastage. This result suggested that the interfering agent might not be the sugar itself but a product of the interaction of sugar with amino acids or peptides. A similar suggestion was also received independently from Dr. Douglas V. Frost of the Abbott Laboratories, namely that the browning reaction might be responsible for the heavier wastage with the sugar-containing hydrolysates. The reader is referred to the discussions by Overby and Frost (15) and Danehy and Pigman (16) of the browning reaction. Although precautions are taken to minimize this interaction in the preparation of commercial hydrolysates containing sugar, no means have been found of avoiding it entirely. As far as oral feeding is concerned, no unfavorable nutritional consequences could be observed (15) for the extent of interaction ordinarily obtained. The following experiment shows that the situation is quite different when administration is by vein.

A given subject was infused on different days with a) a fibrin hydrolysate: glucose combination freshly mixed, and with b) the same solution autoclaved in a standard way. The results illustrated in Figure 5 for a normal subject show that the autoclaving is responsible for the high wastage. The same result was obtained for three convalescent patients.

Clearly the higher wastage of bound amino acids obtained with commercial hydrolysates containing sugar is due almost entirely to products of

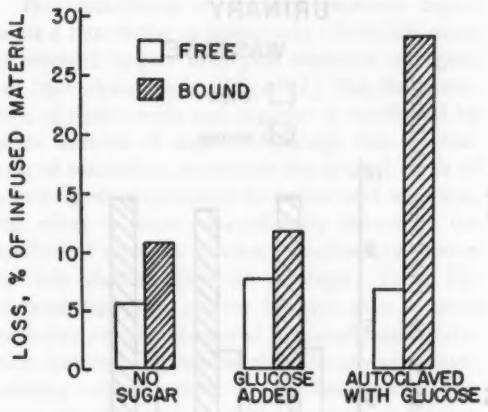

FIG. 5. URINARY PEPTIDE WASTAGE STIMULATED BY AUTOCLAVING WITH SUGAR RATHER THAN BY THE PRESENCE OF SUGAR (NORMAL SUBJECT)

Glucose added to the equivalent of 10 per cent in a 5 per cent fibrin hydrolysate.

chemical reaction between the sugar and constituents of the hydrolysate. These wasted conjugates should not be supposed, however, to be products of interactions between sugar and free amino acids; autoclaving actually produced no perceptible increase in the proportion of the amino acids in the conjugated state (Table I). Another alternative was that an extensive reaction had occurred with the peptides during autoclaving, so as to make about 25 per cent of them unmetabolizable. This possibility is unlikely because the titration curves (Figure 6) of glucose-fortified hydrolysates obtained before and after autoclaving were entirely superimposable, not varying by more than 0.03 pH unit at any point. Reaction of the amino or carboxyl or other dissociating groups of the peptides should lead to a substantial change in the titration curve, especially between pH 4 and 8. Accordingly it is considered likely that small amounts of products of interaction between sugar and amino acids or peptides are interfering with the metabolism of substantial amounts of peptide. The smaller peptide wastage with fructose probably results from the weaker browning reaction which this sugar gives.

The grossly incomplete utilization of infused peptides has suggested that they were used either after extracellular cleavage or after passive diffusion into the cells (14) followed by cleavage there. The present results may suggest a step in their utilization which is affected by other metabolic events. On the other hand perhaps the interaction products instead merely inhibit the cleavage of the peptides.

The extra losses of the *free* amino acids in contrast to the peptides were obtained in every case whether the mixtures were autoclaved or not, and appear to be due mainly to the presence of the sugar itself.

Decreased immediate excretion of nitrogen when sugars are infused first

The foregoing evidences of interference of the sugar with the utilization of the free and combined amino acids of the hydrolysate when the two were given together led us to try infusing the sugar solution first. This was given as 1 liter of a 10 per cent solution at the same standard rate used above; sugar-free fibrin hydrolysate was then infused without the slightest interruption of the flow into the vein. When the sugars were given in this way, they produced no increase whatever in

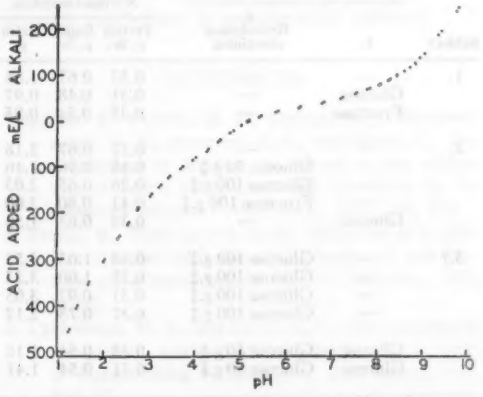

FIG. 6. TITRATION BEHAVIOR BEFORE AND AFTER AUTOCLAVING OF A PARTIAL HYDROLYSATE OF FIBRIN MADE TO 10 PER CENT IN GLUCOSE

The conditions of autoclaving were those described in the experimental section. Beginning at the lower left the even-numbered points (second, fourth, *etc.*) represent the unautoclaved solution, the odd-numbered points, the autoclaved solution. Correction has been made for a shift of the titration curve in the acid direction by 5 milliequivalents per liter during the autoclaving.

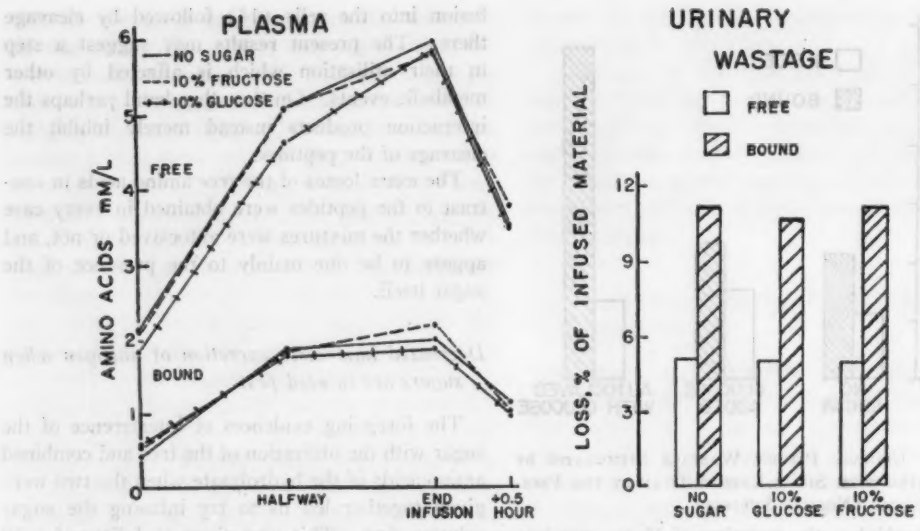

FIG. 7. EXTRA WASTAGE OF AMINO ACIDS AND PEPTIDES AVOIDED BY INFUSING SUGAR SOLUTION PRIOR TO FIBRIN HYDROLYSATE (NORMAL SUBJECT)

TABLE II

Decreased immediate amino acid catabolism when a sugar is infused before the hydrolysate *

Subject	Infusions 1.	Infusions 2. Hydrolysate containing	Nitrogen excretion Pretest g./hr.	Nitrogen excretion Exptl. g./hr.	Nitrogen excretion Extra g.
1.	—	—	0.33	0.67	1.85
	Glucose	—	0.31	0.48	0.97
	Fructose	—	0.38	0.54	0.85
2.	—	—	0.27	0.67	2.18
	—	Glucose 50 g.‡	0.48	0.96	2.46
	—	Glucose 100 g.‡	0.26	0.65	2.03
	—	Fructose 100 g.‡	0.41	0.80	2.00
	Glucose	—	0.33	0.63	1.50
3.†	—	Glucose 100 g.‡	0.40	1.03	3.53
	—	Glucose 100 g.‡	0.37	1.00	3.23
	—	Glucose 100 g.‡	0.31	0.92	3.05
	—	Glucose 100 g.‡	0.37	0.75	2.12
	Glucose	Glucose 50 g.§	0.35	0.54	1.16
	Glucose	Glucose 50 g.§	0.31	0.54	1.41

* Normal subjects; infusion No. 1 (if any) from 8 to 10 A.M., 1 liter of 10 per cent sugar; second infusion from 10 A.M. to 12 M., 1 liter of 5 per cent hydrolysate with or without sugar as shown. Infusions not on consecutive days except for subject 3.

† Six successive days during which subject received a constant oral intake representing 2.4 g. N and 1600 (first 4 days) or 1,500 calories (last 2 days) between the hours of 4 P.M. and 9 P.M. Infusions between 9 A.M. and 12 M. The successive daily N balances in grams were as follows: −4.2; −4.1; −2.2; −2.6; −0.95; −0.14.

‡ Autoclaved with sugar by Abbott Laboratories.

§ Added sterilely just before infusion.

the urinary wastage of amino acids and peptides (Figure 7).

Table II shows the background rates of total nitrogen excretion during a pretest period (between 2 and 8 A.M. for subjects 1 and 2; between 3 P.M. and 8 A.M. for subject 3); and the higher rate during the hydrolysate infusion and the succeeding three hours (referred to as the experimental period). The "extra" nitrogen is the additional amount eliminated during this period of approximately five hours because of the faster rate of excretion. Urine collected during the No. 1 (sugar) infusion was not included in either period. Extra urinary total nitrogen excretion was little modified by inclusion of the sugar in the hydrolysate, but was substantially decreased by giving either sugar in advance. This diminution was highly significant statistically (P was well below 0.001, comparing all infusions not preceded by sugar with all those preceded by sugar). Analyses over an additional four-hour period did not modify the conclusion that the prior infusion of sugar has a much greater effect than the simultaneous infusion in slowing the catabolism of amino acids. The conclusion is also unchanged if the rates of nitrogen excretion during the experimental periods are compared without reference to the background rates.

This retardation of nitrogen excretion represents a retardation of amino acid catabolism, since it involved largely urea plus ammonia nitrogen; we have already seen (Figure 7) that the excretion of amino acids and peptides is unaffected by prior infusion of sugar. Although this retardation of catabolism represents the desired effect of a calorigenic supplement to amino acid nutrition, the effect is more conventionally shown by the method of nitrogen balance. Preliminary studies by this method (see for example, Table II) showed that prior glucose infusion gave as good or better results, compared to simultaneous infusion, but they emphasized the difficulty of demonstrating advantageous or disadvantageous features by this method with the human subject. The requirements for nitrogen balance study with the human are not easily attained, because positivity of nitrogen balance depends not only on the nature of the nutrients received but also on the magnitude of the existing nitrogen deficiency and on other preexisting catabolic or anabolic trends which vary enormously from patient to patient and which change from day to day for a given patient.

Practical considerations

Probably *both prior* and *simultaneous* infusion of sugar should be recommended, if one bears in mind that as many calories as presently possible should be supplied to a patient receiving amino acids by vein. Two reservations, however, are made about the simultaneously infused portion:

a) The present results indicate that the sugar probably should not be autoclaved with the hydrolysate. Storage of sugar and hydrolysate together also are known to cause browning. There is no evidence that this reservation applies to peptide-free hydrolysates.

b) The content in glucose probably should not be as high as 10 per cent, at least for the present rate of infusion.

Wherever autoclaving of a hydrolysate with a sugar seems inescapable, advantage may be taken of the lower reactivity of fructose. It remains to be seen whether fructose has any superiority to glucose when autoclaving is avoided and when the sugar infusion is started prior to the hydrolysate.

SUMMARY

1. The presence of 10 per cent fructose, 5 per cent glucose, or 10 per cent glucose in commercial partial hydrolysates of protein increases substantially the wastage of peptides and amino acids consequent to intravenous infusion. The effect increases in the order named.

2. The extra peptide wastage is associated with higher peptide levels in the plasma, indicating that the disturbance of peptide utilization is prerenal.

3. The extra amino acid wastage is not associated with higher plasma amino acid levels, and accordingly appears to be mainly renal in origin.

4. Slightly higher plasma amino acid levels are generally obtained during infusion of fructose-containing than glucose-containing hydrolysates.

5. The extra peptide wastage is avoided, if glucose is added sterilely immediately before infusion, but reappears if the mixture is autoclaved under usual conditions.

6. Although the presence of sugar in a commercial fibrin hydrolysate increases amino acid and peptide loss, it does not diminish significantly the immediate excretion of total nitrogen; on the other hand if the sugar (glucose or fructose) is infused just before the hydrolysate, the immediate nitrogen catabolism is substantially reduced.

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THYROID FUNCTION ASSAY WITH RADIOIODINE. I. PHYSICAL BASIS OF STUDY OF EARLY PHASE OF IODINE METABOLISM AND IODINE UPTAKE[1]

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The factors which limit the diagnostic accuracy of radioactive iodine tracer studies may be summarized as follows:

1. There is probably a continuous gradation between the normal and the abnormal with no sharp borderlines between, so that it may not always be possible to establish abnormality.
2. The tests usually attempt to quantitate uptake of ^{131}I by the gland, or percentage conversion of inorganic ^{131}I to protein bound iodine, but do not actually assay whether or not the thyroid is secreting the hormone in required amounts.
3. There may be present quite large experimental errors.
4. Unknown dietary or other factors may cause confusion.

The first two factors will not be rectified by good experimental accuracy, but some enhancement of diagnostic accuracy in available tests will assist materially in the overall evaluation of the problem.

Although many different types of radioiodine tests have been described (1), the scope of this paper is restricted to the early period up to 24 hours after injection of the tracer dose. Technical improvements would be of particular benefit if applied to observations made in this early period, because such tests give an earlier decision. An early thyroid uptake test of this type has been described by Haigh and his coworkers (2–4), and much improved versions more recently by Berson, Yalow, Sorrentino, and Roswit (5), and Clarke and Aujard (6). Loverdo (7) also uses a similar technique, but gives no diagnostic results.

Berson, Yalow, Sorrentino, and Roswit's (5) excellent method covers the period up to 30 minutes from the time of injection, and it has seemed well worthwhile to extend this method to cover a variable period up to 24 hours after administration. These longer times allow a more accurately measurable amount of radioactivity to accumulate in the thyroid gland when the rate of uptake is small. Small uptake rates occur in hypothyroid cases and also in some patients with cardiac disease.

A major difficulty in the early thyroid uptake test arises from the need for a close estimate of the effect of radioiodine in the neck tissues other than the thyroid gland. These tissues may contribute over half the total *in vivo* counts over the neck in the early stages of uptake measurements, not only on cardiac or hypothyroid patients, but also on many normal subjects. It is quite common practice to measure the counting rate over the thigh and to assume that this is equal to the rate over the neck tissues, but we have been unable to correlate these two counts closely enough. Accordingly, our approach has been first to study the variation of counts over the neck in a number of test cases and then to try to reduce these counts by careful collimation to a level low enough to enable average values to be applied for all patients.

The matters requiring preliminary study, then, are the uncertainty persisting in the early metabolism of iodine, and the manner of its movement in the neck tissues; the first part of this paper describes the mathematical and experimental basis for the investigation of these particular problems; and the second part includes a description of the routine simplified test methods finally developed.

METABOLIC RELATIONS

Choice of metabolic schemes

The metabolic course followed by a tracer dose of iodide placed initially in the human blood stream has been dis-

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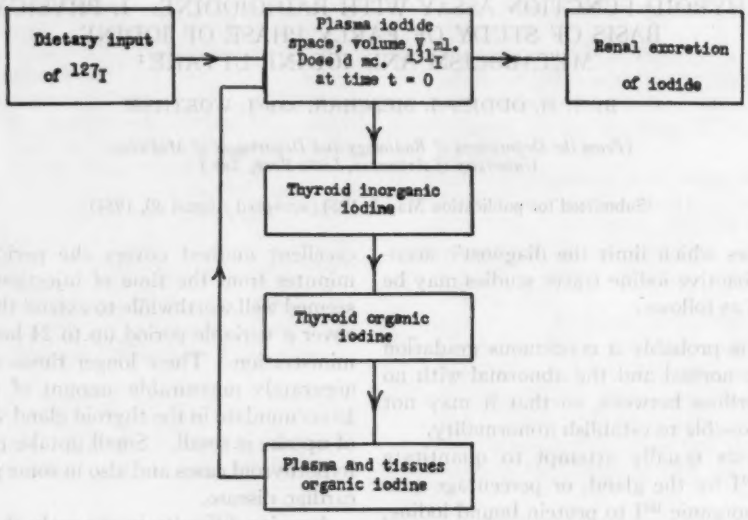
Dietary input of ^{127}I
Plasma iodide space, volume V ml. Dose d mc. ^{131}I at time t = 0
Renal excretion of iodide
Thyroid inorganic iodine
Thyroid organic iodine
Plasma and tissues organic iodine

FIG. 1. COMPLETE APPROXIMATE SCHEME OF IODINE METABOLISM

cussed previously on a mathematical basis by several writers (1, 8–12); the reader is referred to a recent review by Riggs (1) for a full discussion of this subject. The overall metabolic scheme is generally agreed to be as shown in Figure 1, provided some minor simplifying assumptions are made, chiefly as follows:

1. The initial tracer dose (d mc.) mixes with negligible delay at zero time (t = 0) with the initial volume of blood plasma (V_0 ml.) throughout the circulatory system.

2. Losses of radioiodine through respiration, or *via* saliva, sweat or feces are neglected.

3. Iodine may be assumed not to be reabsorbed from urine in the bladder.

The equivalent volume of the "iodide space" (V ml.) through which the circulating radioiodide is distributed, varies with time, increasing from V_0 at time t = 0. Thus the complete metabolic equations, to account for all the variables over a large range of time, involve too many exponential terms to be evaluated with any degree of accuracy in practice. The problem can, however, be studied in sections by making further more critical assumptions; in several earlier analyses (1, 9, 10, 12) V was assumed constant, so that the equations developed account approximately for the behaviour of the test dose in the later metabolic stages, but they are not sufficiently accurate for the early phase. On the other hand, Brownell's analysis (8, 11) deals with the early phase, prior to the slow output of appreciable amounts of organically bound iodine from the thyroid gland, by breaking up V into a two-compartment system as shown in Figure 2,

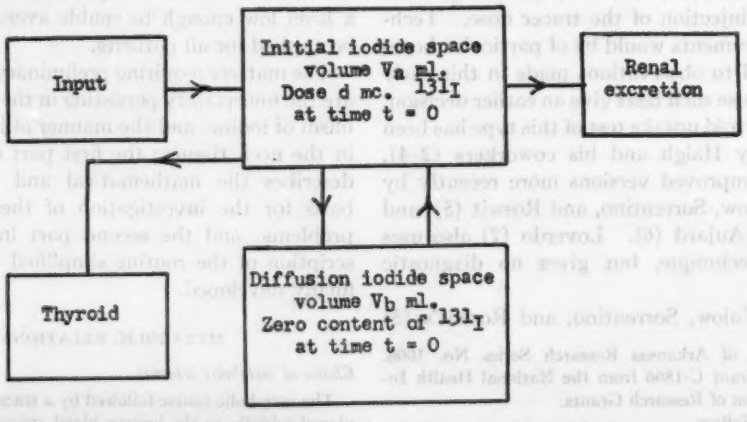
Input
Initial iodide space volume V_a ml. Dose d mc. ^{131}I at time t = 0
Renal excretion
Thyroid
Diffusion iodide space volume V_b ml. Zero content of ^{131}I at time t = 0

FIG. 2. BROWNELL'S SCHEME FOR EARLY PHASE OF IODINE METABOLISM

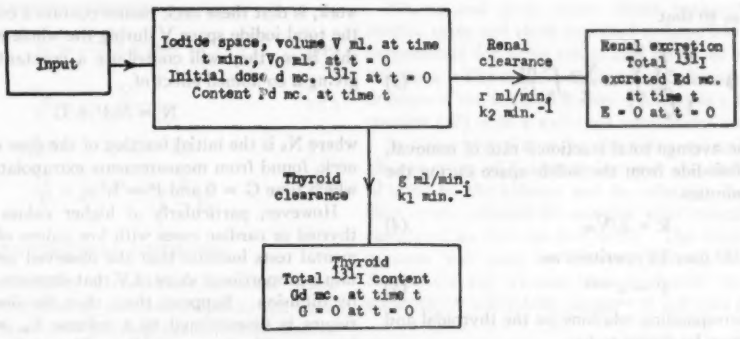
Input
Iodide space, volume V ml. at time t min., V0 ml. at t = 0
Initial dose d mc. 131I at t = 0
Content Pd mc. at time t
Renal clearance
r ml/min, k2 min.-1
Renal excretion
Total 131I excreted Ed mc. at time t
E = 0 at t = 0
Thyroid clearance
g ml/min, k1 min.-1
Thyroid
Total 131I content
Gd mc. at time t
G = 0 at t = 0

FIG. 3. SCHEME DISCUSSED IN THIS PAPER FOR EARLY PHASE OF IODINE METABOLISM

where diffusion takes place back and forwards between V_a and V_b. The resulting equations are undoubtedly closer to the truth than those with constant V, but unfortunately we have been able to make them fit neither our own experimental measurements nor the data on V published by Berson, Yalow, Sorrentino, and Roswit (5). It seems that a multi-compartment diffusion system is really required for the iodide space, and it is, therefore, preferable, in our opinion, to deal with the problem experimentally, making no assumptions about the analytical form for the variation of V with time. In other words, we begin with the scheme of Figure 3 for the early phase of iodide metabolism, before any appreciable tracer has left the thyroid in organic form.

Symbols used

For convenience, the symbols used in the discussions to follow are listed alphabetically below:

a ml./min. = g + r = Combined clearance rate, that is the volume of plasma cleared of iodide per minute by the thyroid and kidneys together.

d mc. = Net amount of ^{131}I injected.

E = Fraction of the dose d excreted by the kidneys up to time t.

G = Fraction of the dose d taken up by the thyroid up to time t.

g ml./min. = Thyroid rate of clearance of iodide from plasma.

k min.$^{-1}$ = $k_1 + k_2$ = Average combined (thyroid plus kidneys) fractional rate of removal of radio-iodide from the iodide space during the time interval t = 0 to 30 minutes; that is, an average fraction k of the circulating iodide pool is removed each minute.

k_1 min.$^{-1}$ = Average fractional rate of uptake by the thyroid from t = 0 to 30 minutes.

k_2 min.$^{-1}$ = Average fractional rate of renal excretion from t = 0 to 30 minutes.

N = Fraction of the dose d observed in the neck by external counting at time t, including both thyroid and extra-thyroid tissues.

N_0 = Initial value of N at time t = 0 assuming instantaneous mixing of the dose with the circulating plasma.

N_2 = Value of N at t = 2 minutes.

$N_{2,10}$ = Average value of N between 2 and 10 minutes.

P = Fraction of the dose d remaining in the iodide space at time t.

p ml.$^{-1}$ = Fraction of the dose d remaining in the plasma iodide space at time t, per unit volume of plasma.

R mc. = Content of the ^{131}I source prepared for purposes of calibration.

r ml./min. = Renal rate of clearance of iodide from the plasma.

t min. = Time from injection of the test dose.

V ml. = Volume of the total apparent iodide space at time t.

V_0 ml. = Initial value at V at t = 0.

V_{30} ml. = Value of V at t = 30 minutes.

$1/\bar{V}_{30}$ ml.$^{-1}$ = Mean value of 1/V from t = 0 to 30 minutes.

V_aN_0 ml. = Apparent volume of iodide space, at time t, that is "observed" in the extra-thyroidal neck tissues by the external counter.

W kg. = Weight of the patient.

$\phi = \bar{V}_{30}$ times the average value of 1/V up to time t.

Basic equations

Considering now the movement of a tracer dose, d mc., after injection at zero time t = 0 as in Figure 3, the basic differential equation for the fraction P remaining in the iodide space V ml. at time t is clearly

$$\frac{dP}{dt} = -\frac{aP}{V} \qquad (1)$$

where a ml. per min. is the combined rate of clearance of plasma (13) obtained by adding together the thyroid and renal clearance rates, g ml. per min. and r ml. per min., respectively.

Now avoiding the assumption of any particular functional form for the variation of V with time, but merely taking V as an unknown function of t, the solution of (1), with P = 1 at t = 0, is

$$P = e^{-a\int_0^t \frac{dt}{V}} \qquad (2)$$

Now define a new function ϕ as the ratio of the average value of 1/V from time 0 to t to the average value up to

30 minutes $1/\overline{V}_{30}$, so that

$$\phi = \frac{30\int_0^t \frac{dt}{V}}{t\int_0^{30} \frac{dt}{V}} = \frac{\overline{V}_{30}}{t}\int_0^t \frac{dt}{V} \qquad (3)$$

and write for the average total fractional rate of removal, k min.$^{-1}$, of radioiodide from the iodide space during the period 0 to 30 minutes

$$k = a/\overline{V}_{30} \qquad (4)$$

Then equation (2) may be rewritten as

$$P = e^{-k\phi t} \qquad (5)$$

Similarly the corresponding relations for the thyroidal and renal fractions may be shown to be

$$G = (1 - e^{-k\phi t})\, k_1/k \qquad (6)$$

$$E = (1 - e^{-k\phi t})\, k_2/k \qquad (7)$$

where k_1 and k_2 min.$^{-1}$ are the average fractional rates of thyroidal uptake and renal removal over the same 30-minute period. There is, of course, no necessity to choose the time of 30 minutes for the definition of ϕ; we have done so because this is a convenient period for some uptake measurements, Berson, Yalow, Sorrentino, and Roswit (5) have already published results of tests made at this particular time after injection, and the choice of $t = 0$ for the reference point leads to no real improvement because V_0 is subject to considerable experimental uncertainty.

The fractional rates k_1 and k_2 are related to the clearance rates by the ratios

$$k_1/k = g/a \qquad (8)$$

$$k_2/k = r/a \qquad (9)$$

The k value so obtained is identical to the disappearance rate in common use, except that a correction has been applied for the changing iodide space instead of assuming it to be constant.

In equilibrium, where the dietary input of stable iodide, Q micrograms per day, equals the urinary output, the amount accumulated is kQ/k_2, or gQ/r micrograms per day, so that it is immaterial whether k values or clearance rates are used as parameters.

However, to utilize clearance rates, we must assume that the actual volumes themselves are constant, or some known function of body weight; whereas, when k values are found, it is assumed only that the *relative* changes of the volume of the iodide space are constant for all patients.

Additional equations for uptake studies

When uptake measurements are made by the usual external *in vivo* counting methods, to account for the observed radioiodine content N of the neck we must add together the thyroid fraction G and some contribution from the blood vessels and other extrathyroid tissues "visible" to the counter. This contribution is by no means negligible when low uptakes are involved, even at $t = 24$ hours.

The simplest assumption to make, and the one that is implicit in Berson, Yalow, Sorrentino, and Roswit's (5) work, is that these neck tissues contain a constant share of the total iodide space V during the whole experiment. If this is so, they will contribute a constant portion of P, giving a combined effect of

$$N = N_0P + G \qquad (10a)$$

where N_0 is the initial fraction of the dose observed in the neck, found from measurements extrapolated to $t = 0$, at which time $G = 0$ and $P = 1$.

However, particularly at higher values of t in hypothyroid or cardiac cases with low values of k, our experimental tests indicate that the observed neck tissues contain a proportional share of V that decreases as V increases by diffusion. Suppose, then, that the share of the neck tissues is proportional to a volume V_n, which does not increase with time as rapidly as V, and that $V_n = V = V_0$ at $t = 0$.

Then writing equation (10a) in the modified form

$$N = PN_0V_n/V + G \qquad (10b)$$

and using (5) and (6) it follows that

$$N = N_0V_ne^{-k\phi t} + (1 - e^{-k\phi t})\, k_1/k \qquad (11)$$

Since

$$k = k_1 + k_2 \qquad (12)$$

equations (7) and (11) may be solved[3] to give

$$k_1/k = \frac{N - N_0V_n/V + EN_0V_n/V}{N - N_0V_n/V + E} \qquad (13)$$

and

$$k\phi t = \ln \frac{k_1/k - N_0V_n/V}{k_1/k - N} = \ln \frac{1 - N_0V_n/V}{1 - N - E} \qquad (14)$$

Further, from the definition (3)

$$\frac{1}{V} = \frac{d(\phi t)}{dt}(1/\overline{V}_{30}) = \frac{p}{P} = pe^{k\phi t} \qquad (15)$$

where p ml.$^{-1}$ is the fractional concentration of the dose remaining in the plasma.

In the simple case where $V_n = V$ the above relations should theoretically yield values of the unknowns k_1, k_2 and V when a set of precise observations of N, E, and P is available.

In the more general case when $V_n \neq V$, the problem is not so easy. A possible line of approach, primarily to find V_n/V, is to conduct a series of observations after the thyroid uptake has been made substantially zero ($k_1 = 0$) by the administration of large doses of stable ^{127}I iodide.

[3] From (7) and (12) it follows that

$$k_1/k = 1 - k_2/k = \frac{1 - e^{-k\phi t} - E}{1 - e^{-k\phi t}}$$

and from (11)

$$k_1/k = \frac{N - N_0(V_n/V)e^{-k\phi t}}{1 - e^{-k\phi t}} \qquad (13a)$$

so that

$$N - N_0(V_n/V)e^{-k\phi t} = 1 - E - e^{-k\phi t}$$

and

$$e^{-k\phi t} = \frac{1 - N - E}{1 - N_0V_n/V} \qquad (14a)$$

Equation (14) follows directly from (14a) and (13) is derived by substituting (14a) in (13a).

For this series the equations become

$$N = \frac{N_0 V_n}{V} e^{-k_2 \phi t} \quad (16)$$

$$\frac{V_n}{V} = \frac{N}{N_0(1 - E)} \quad (17)$$

$$k_2 \phi t = -\ln (1 - E) \quad (18)$$

$$\frac{1}{V} = p e^{k_2 \phi t} = \frac{p}{1 - E} \quad (19)$$

EXPERIMENTAL METHODS

To test the applicability of the above formulae careful measurements are needed of the radioiodine in the neck, the plasma and the urine of selected patients over most of the period before radioactive hormone is released by the thyroid.

Various methods of making these measurements have been thoroughly described by others (13–16); our own arrangements closely resemble those of Berson, Yalow, Sorrentino, and Roswit (5) and of Loverdo (7), so they are described only briefly below.

Dose: From ^{131}I, supplied by the Atomic Energy Commission at Oak Ridge, a carrier-free dose D mc. and a reference standard R mc. are prepared, each containing about 0.1 mc. in 5 ml. of solution in similar glass vials. The ratio D/R is carefully measured by gamma counting, and later the residues left behind in the injection syringe and vial are also compared with R so that the net dose d mc. injected can be found as a ratio d/R.

At the same time a further aliquot of the original solution is measured and diluted to serve as a standard for comparison with blood and urine samples.

Uptake measurement: For uptake observations a collimated scintillation counter is used at a long crystal-skin distance (47 cm.) to avoid large "inverse-square" errors. The lead collimator walls are 3.8 cm. (1.5 inch) thick; its axial aperture has a minimum diameter of 1.27 cm. (0.5 inch) and tapers outwards with a half-angle of 7.1° so that a circle of 13.7 cm. diameter is in full view of the crystal at 50 cm. distance. A conical lead plug closes the aperture when background readings are taken. In all our preliminary experiments the axial length of the collimator was 3.8 cm., after which it was extended by an added lead cone to a total length of 20 cm. in order to lower the counts in the neck tissue by eliminating most of the penumbral region viewed by the crystal. These two arrangements will be referred to as the "short" and "long" collimators.

The counter is operated with an E. K. Cole precision ratemeter type N522, and its output is recorded continuously on a Brown type 153 chart-recorder. Recordings are also made for the purpose of calibration with the reference source R at 47 cm. distance, both before and after the *in vivo* observations. Corrections necessary for absorption and backscatter in the neck tissues were found as usual from a series of experiments with wax phantoms of different sizes.

The dose d is given intravenously in a few seconds and the recording equipment or timing clock is started at zero time, which is taken when half the dose has entered the patient.

Plasma and urine assay: Most patients, particularly cardiac cases and those expected to have low k values, are catheterized for urine collections up to 60 minutes after injection. The urine and plasma samples are all expressed in terms of the net dose d after counting in a skirted Geiger counter (17) or in a well-type scintillation counter.

In fixing the time t for the urine collections we have allowed a lag of 5 minutes between the time of excretion of urine by the kidneys and its collection in the bladder; thus urine collected 65 minutes after injection would be calculated as that up to t = 60. The delay period of 5 minutes was used by Berson, Yalow, Sorrentino, and Roswit (5) and we also have confirmed its approximate accuracy by a few tests on some of our own patients.

RESULTS

Using the above methods we have made a series of tests to find the variation with time of the quantities V_n/V, V and ϕ in a number of patients, as follows:

Series A

This series consisted of euthyroid and cardiac cases studied before, and in some cases again after, a course of Lugol's medication, with serial readings of the counting rate over the neck, and blood, and urine radioiodine assays at times t of 0 to 30 minutes, 1, 2, 4, and 24 hours.

It was simplest to analyse the post-Lugol's results first, using equation (17) to find V_n/V, which is recorded as a ratio to the value $(V_n/V)_2$ at time t = 2 minutes rather than t = 0. This is necessary because the injected dose takes about one minute to mix with the circulating blood and the chart recorder trace shows an initial peak from the concentrated dose moving up the patient's arm and past the field of view of the counter. This peak, which is, of course, much larger with the short collimator than with the long one, must be allowed to die away quickly by using a short time-constant in the ratemeter circuit: one of our experimental cases in this series was spoilt for this reason, the time-constant control being switched too early to the 20-second setting, thus making the recorded rate too high at t = 2 minutes. Excluding this case, we have measured five others with the results shown in Figure 4. At t = 24 hours we have found, for a total of eleven low-uptake cases, a mean value of $\frac{V_n/V}{(V_n/V)_2} = 0.52 \pm 0.18$(S.D.). Of these cases, four showed positive edema and the other seven were nonedematous. The range of values was

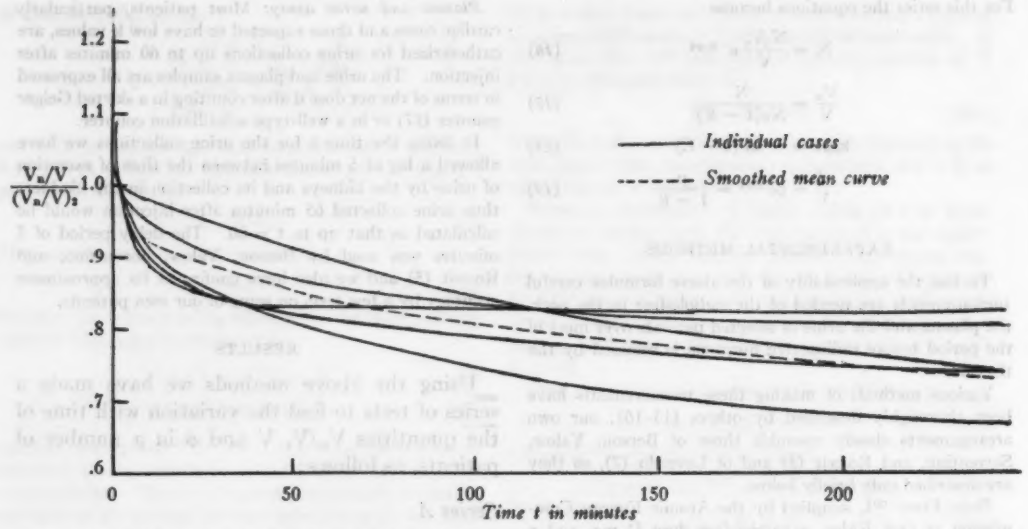

FIG. 4. EFFECTIVE NECK VOLUME RATIO FOUND IN FIVE EXPERIMENTAL PATIENTS WHOSE THYROID GLANDS WERE SATURATED WITH LUGOL'S SOLUTION

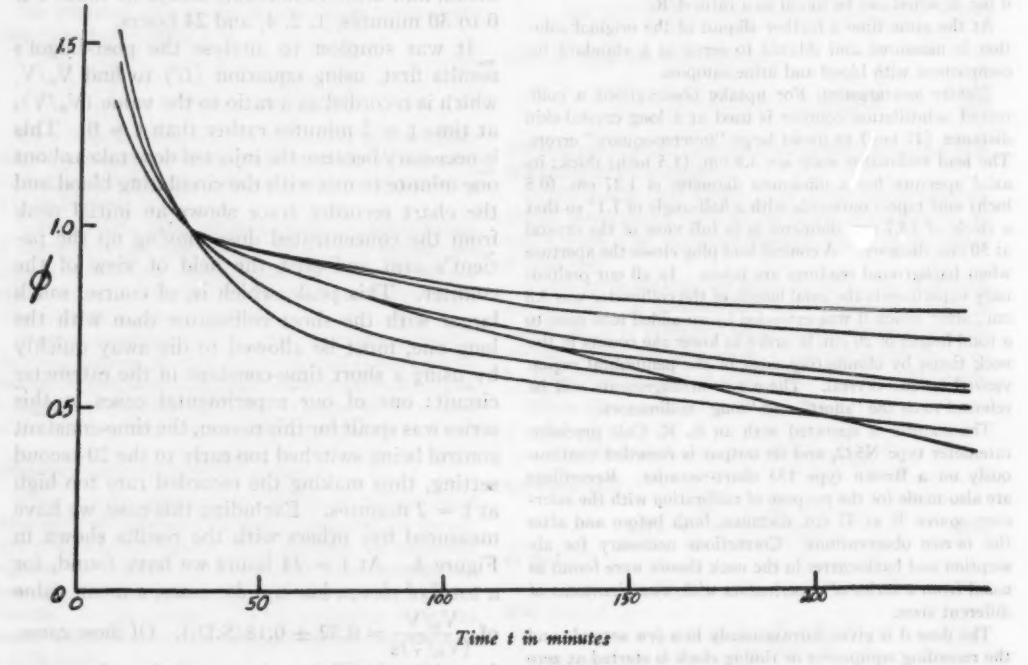

FIG. 5. EXPERIMENTAL CURVES FOR ϕ
FIG. 5A. RESULTS FOR LOW K VALUES, SERIES A

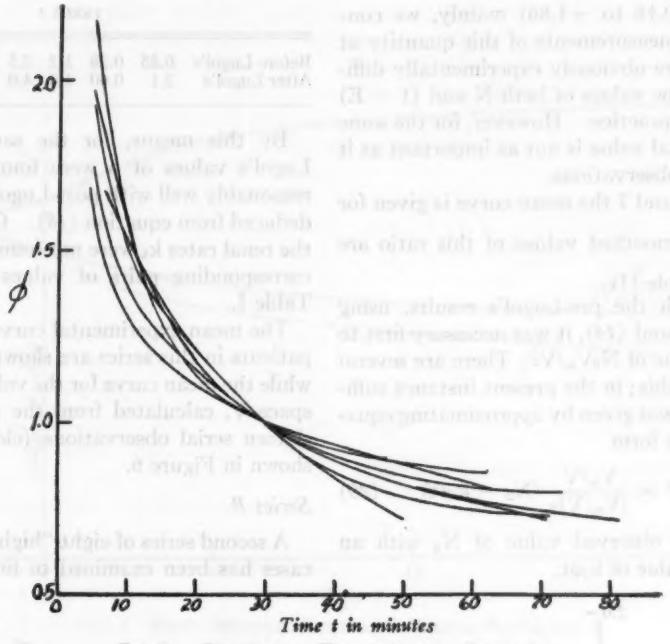

FIG. 5B. RESULTS FOR HIGH K VALUES, SERIES B

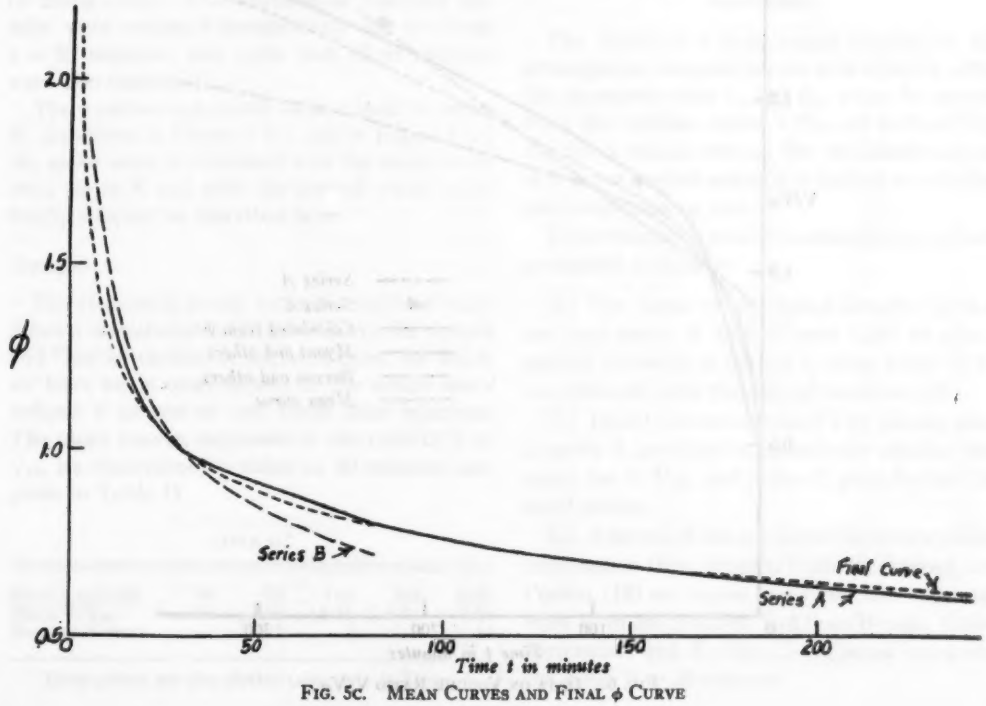

FIG. 5C. MEAN CURVES AND FINAL φ CURVE

wide (from −0.16 to +1.86) mainly, we consider, because measurements of this quantity at t = 24 hours are obviously experimentally difficult with the low values of both N and (1 − E) encountered in practice. However, for the same reason the actual value is not as important as it is with earlier observations.

In Figures 4 and 7 the mean curve is given for $\frac{V_n/V}{(V_n/V)_2}$ and smoothed values of this ratio are included in Table III.

To deal with the pre-Lugol's results, using equations (*13*) and (*14*), it was necessary first to deduce the value of N_0V_n/V. There are several ways of doing this; in the present instance sufficient accuracy was given by approximating equation (*11*) to the form

$$N_0V_n/V = \frac{V_n/V}{(V_n/V)_2}(N_2 - k_1\phi t) \qquad (20)$$

and using the observed value of N_2 with an approximate value of $k_1\phi t$.

TABLE I

Before Lugol's	0.85	0.28	1.2	3.5	1.8	$\times 10^{-3}$ min.$^{-1}$
After Lugol's	2.1	0.60	1.0	4.0	1.2	$\times 10^{-3}$ min.$^{-1}$

By this means, for the same patient, pre-Lugol's values of ϕ were found, which agreed reasonably well with post-Lugol's values already deduced from equation (*18*). On the other hand the renal rates k_2 were not found to be the same, corresponding pairs of values being shown in Table I.

The mean experimental curves for ϕ for seven patients in this series are shown in Figure 5 (a), while the mean curve for the volume of the iodide space V, calculated from the plasma assays in sixteen serial observations (eleven patients), is shown in Figure 6.

Series B

A second series of eight "high-k" hyperthyroid cases has been examined to find the early part

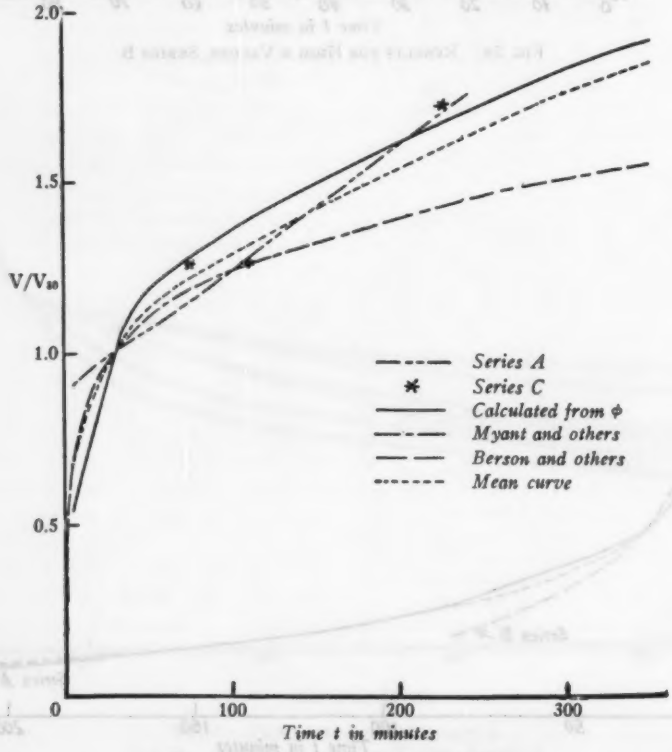

FIG. 6. DATA ON VOLUME RATIO V/V_∞

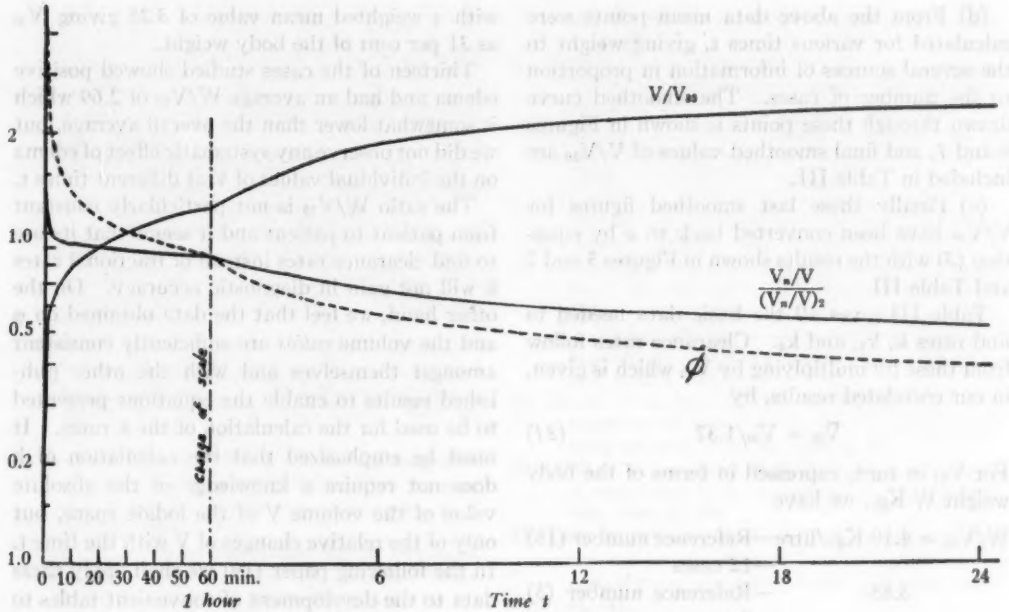

FIG. 7. FINAL SMOOTHED CURVES FOR VOLUME RATIOS AND ϕ

of the ϕ curve. Observations of the neck uptake were recorded continuously up to about t = 60 minutes, and urine and blood samples were also measured.

The ϕ values, calculated as described for series A, are shown in Figure 5 (b), and in Figure 5 (c) the mean value is compared with the mean curve from series A and with the overall mean curve finally adopted as described later.

Series C

The remaining group we have examined comprises a miscellaneous group of thirty-six normal and "low-k" cardiac or athyroid cases, for which we have made observations of the iodide space volume V at one or two times after injection. The mean results, expressed as the ratio of V to V_{30}, its instantaneous value at 30 minutes, are given in Table II.

TABLE II *

Mean t minutes	30	76	110	228	1440
Mean V/V_{30}	1.00	1.26	1.26	1.71	2.01
Number of cases	—	25	2	3	14

* These points are also plotted separately in Figure 6.

DISCUSSION

The quantity ϕ is of major interest in this investigation because errors in ϕ directly affect the measured rates k_1 and k_2; ϕ can be derived from the volume ratios V/V_{30} as well as from the direct measurements, but the absolute value of V is not needed unless it is desired to calculate clearance rates (g and r).

To correlate the available information we have proceeded as follows:

(a) The mean results found directly for ϕ in our test series A and B were used to give a smooth curve for ϕ against t, from which V/V_{30} was deduced with the help of equation (*15*).

(b) Direct measurements of V by plasma assay in series A provided independently another time curve for V/V_{30}, and series C gave further isolated points.

(c) A search of the published literature yielded information from Myant, Corbett, Honour, and Pochin (18) on twelve cases over the range of t from 30 to 360 minutes, and from Berson, Yalow, Sorrentino, and Roswit (5) on seven cases over the range 0 to 30 minutes.

(d) From the above data mean points were calculated for various times t, giving weight to the several sources of information in proportion to the number of cases. The smoothed curve drawn through these points is shown in Figures 6 and 7, and final smoothed values of V/V_{30} are included in Table III.

(e) Finally these last smoothed figures for V/V_{30} have been converted back to ϕ by equation (*3*) with the results shown in Figures 5 and 7 and Table III.

Table III gives all the basic data needed to find rates k, k_1, and k_2. Clearance rates follow from these by multiplying by ∇_{30} which is given, in our correlated results, by

$$\nabla_{30} = V_{30}/1.32 \qquad (21)$$

For V_{30} in turn, expressed in terms of the body weight W Kg., we have

W/V_{30} = 4.10 Kg./litre	—Reference number (18) —12 cases
3.85	—Reference number (5) —7 cases
2.92	—This paper—44 cases

TABLE III

Mean smoothed values of various factors for uptake calculations *

t minutes	V/V_∞	ϕ	ϕt	$\frac{V_\infty/V}{(V_\infty/V)_3}$
0	(0.18)	(4.2)	0.0	(1.34)
2	0.58	2.15	4.3	1.00
5	0.68	1.58	7.9	0.95
10	0.78	1.31	13.1	0.92
15	0.85	1.18	17.7	0.91
20	0.90	1.10	22.0	0.90
30	1.00	1.00	30.0	0.88
40	1.08	0.932	37.3	0.87
50	1.13	0.885	44.2	0.86
60	1.17	0.846	50.8	0.84
70	1.20	0.816	57.1	0.83
80	1.23	0.792	63.4	0.82
90	1.26	0.772	69.5	0.81
100	1.29	0.754	75.4	0.80
120	1.34	0.725	87.0	0.79
140	1.39	0.700	98.0	0.78
160	1.44	0.679	109	0.77
180	1.49	0.661	119	0.76
200	1.53	0.645	129	0.75
240	1.63	0.617	148	0.72
300	1.76	0.582	175	0.69
360	1.86	0.555	200	0.66
480	2.00	0.515	247	0.62
600	2.11	0.486	292	0.59
720	2.20	0.463	333	0.57
1080	2.36	0.419	453	0.54
1440	2.45	0.393	566	0.52

* For interpretation of symbols, see Text.

with a weighted mean value of 3.25 giving V_{30} as 31 per cent of the body weight.

Thirteen of the cases studied showed positive edema and had an average W/V_{30} of 2.69 which is somewhat lower than the overall average, but we did not observe any systematic effect of edema on the individual values of V at different times t.

The ratio W/V_{30} is not particularly constant from patient to patient and it seems that its use to find clearance rates instead of fractional rates k will not gain in diagnostic accuracy. On the other hand, we feel that the data obtained on ϕ and the volume *ratios* are sufficiently consistent amongst themselves and with the other published results to enable the equations presented to be used for the calculation of the k rates. It must be emphasized that the calculation of k does not require a knowledge of the absolute value of the volume V of the iodide space, but only of the relative changes of V with the time t. In the following paper (19) we shall apply these data to the development of convenient tables to simplify the routine measurement and calculation of uptake rates, and in a later publication an attempt will be made to correlate diagnostic limits deduced from the published work of others.

SUMMARY

The early metabolism of iodide in the human body is discussed on a mathematical basis with the object of improving the accuracy of diagnostic tests of thyroid conditions with radioiodine. Allowance is made for changes in the iodide space and the effective volume of neck tissues observed by external gamma ray counting at various times after the administration of the test dose. After testing the resulting equations by experiments on a series of patients, the mean test results are combined with data deduced from the publications of other workers to give tabulated information suitable for practical use.

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THYROID FUNCTION ASSAY WITH RADIOIODINE. II. ROUTINE CALCULATION OF THYROIDAL AND RENAL RATE FACTORS[1]

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(Submitted for publication May 7, 1954; accepted August 30, 1954)

In the preceding paper (1) mathematical equations and numerical data were presented describing the early movement of a test dose of radioiodine in the human body. The information given can be used directly to calculate thyroidal uptake and renal excretion rate factors (or clearance rates) from observed values of the fractions of the net dose in the neck, N, and urine E, without the need for any blood samples, but the process is rather too tedious for routine use. However, the procedure can be greatly simplified by making suitable charts relating N and E to the thyroidal, k_1, and renal, k_2, rate factors for selected *fixed* times t after injecting the test dose. Fixed time schedules are necessary because otherwise a large number of charts is required. For convenience the pertinent equations from the preceding paper (1) are repeated below.

$$k = k_1 + k_2 \qquad (1)$$

$$k_1/k = \frac{N - N_0V_n/V + EN_0V_n/V}{N - N_0V_n/V + E} \qquad (2)$$

$$k\phi t = \ln \frac{1 - N_0V_n/V}{1 - N - E} \qquad (3)$$

All the symbols used are as defined in the preceding paper (1), namely:

E = The fraction of the original dose excreted by the kidneys up to time t minutes after injection.

k min.$^{-1}$ = The average combined (thyroid plus kidneys) fractional rate of removal of radio-iodide from the iodide space during the time interval t = 0 to 30 minutes; that is, an average fraction k of the circulating iodide pool is removed each minute.

k_1 min.$^{-1}$ = The average fractional rate of uptake by the thyroid from t = 0 to 30 minutes.

k_2 min.$^{-1}$ = The average fractional rate of renal excretion from t = 0 to 30 minutes.

N = The fraction of the original dose observed in the neck by external counting at time t, including both thyroid and extra-thyroid tissues.

N_0 = The initial value of N at time t = 0 assuming instantaneous mixing of the dose with the circulating plasma.

t min. = The time from injection of the test dose.

V ml. = The volume of the total effective iodide space at time t.

V_nN_0 ml. = The apparent volume of iodide space, at time t, that is "observed" in the extra-thyroid neck tissues by the external counter.

ϕ = The average value of 1/V up to time t expressed as a fraction of the average up to t = 30 minutes.

The above three equations are all that are needed for the development of simple routine methods and charts, as will be shown in the following sections.

EXTRA-THYROIDAL RADIOIODINE CONTENT OF NECK TISSUES OBSERVED BY EXTERNAL COUNTER

The term N_0V_n/V in equations (*2*) and (*3*) is derived from the fraction of the net dose observed in the extra-thyroidal neck tissues at time t, which has been shown (1) to be given by $\frac{N_0V_ne^{-k\phi t}}{V}$. It has also been shown that V_n/V varies with time, but average smoothed values of this ratio have been tabulated in the form $\frac{V_n/V}{(V_n/V)_2}$—where the denominator represents the value of V_n/V at exactly two minutes after injection.

If then, N_0V_n/V (at any particular time t) can be treated as a *constant* for all patients, equations (*1*), (*2*) and (*3*) can be represented on only one pair of charts connecting k_1 and k_2 with N and E for each selected time t; but if N_0V_n/V varies too widely from patient to patient, a large number of pairs of charts must be constructed to cover the range of variation of this factor. Moreover, there is the additional advantage that if N_0V_n/V is known with sufficient accuracy for all patients, there will usually be no need to make an initial *in vivo* count over the neck immediately after injection.

Obviously variations from patient to patient

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[2] Fulbright Fellow.

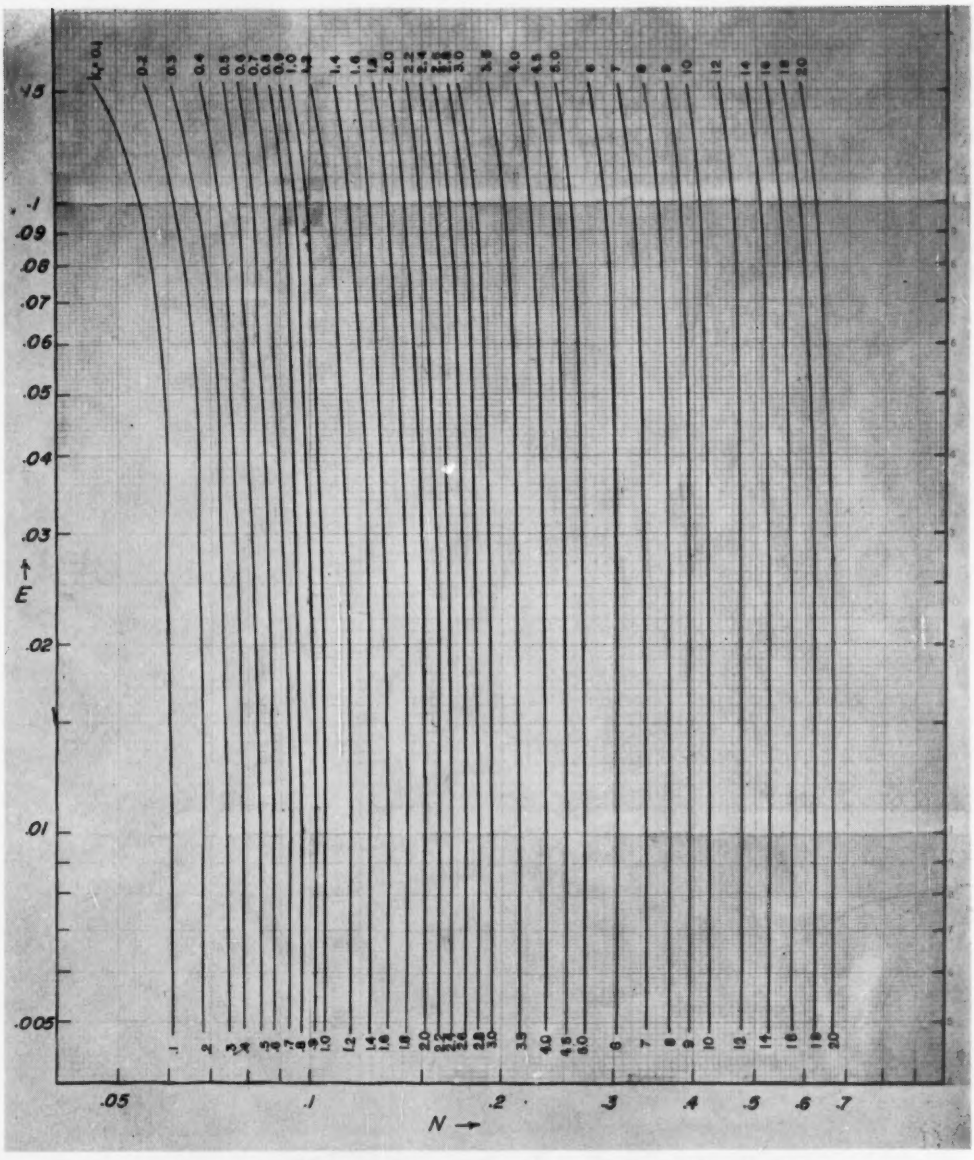

FIG. 1. CHART FOR THYROIDAL RATE k_1 MEASURED AT ONE HOUR ($t = 60$)

become less troublesome when the extra-thyroidal tissue contribution N_0V_n/V is kept small by careful collimation of the external counter; to provide some data on this point we have measured the value of $N_0(V_n/V)_2$—that is the extra-thyroidal neck tissue fraction two minutes after injection—in the same athyroid patient with four different counting arrangements with the results presented in Table I.

The benefit derived from using a long collimator is apparent from the reduced value of the contribution of the neck tissues. In addition we have made observations on two series of different patients with long and short collimators, finding

mean values with standard deviations of the individual values set out in Table II.

Here again the long collimator gives more concordant results, and by using it for routine uptake studies $N_0(V_n/V)_2$ can be taken as 0.071, with a *maximum* expected variation of about $\pm.027$, for all cases except doubtful ones undergoing repeated tests; such cases occur sometimes when the numerical results of a first test lie close to the diagnostic borderline values separating euthyroid and hyperthyroid groups.

For the latter exceptional cases, or for collimators of different shape, it is necessary to deduce the individual values of N_0V_n/V from the initial and final total counts over the neck. This can be done by approximating equation (*11*) of the preceding paper for times t and two minutes, finally obtaining the approximate relation

$$N_0V_n/V = [\phi t N_2 - 4.3\,N] \Big/ \left[\frac{\phi t(V_n/V)_2}{V_n/V} - 4.3\right] \quad (4)$$

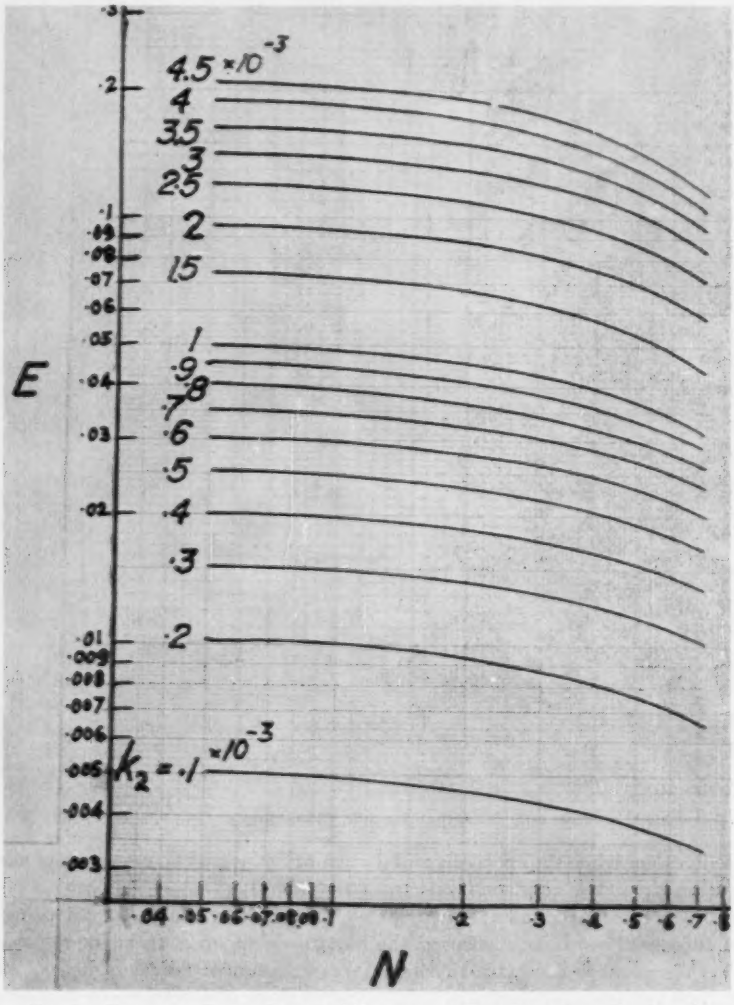

FIG. 2. CHART FOR RENAL RATE k_2 MEASURED AT ONE HOUR ($t = 60$ *minutes*)

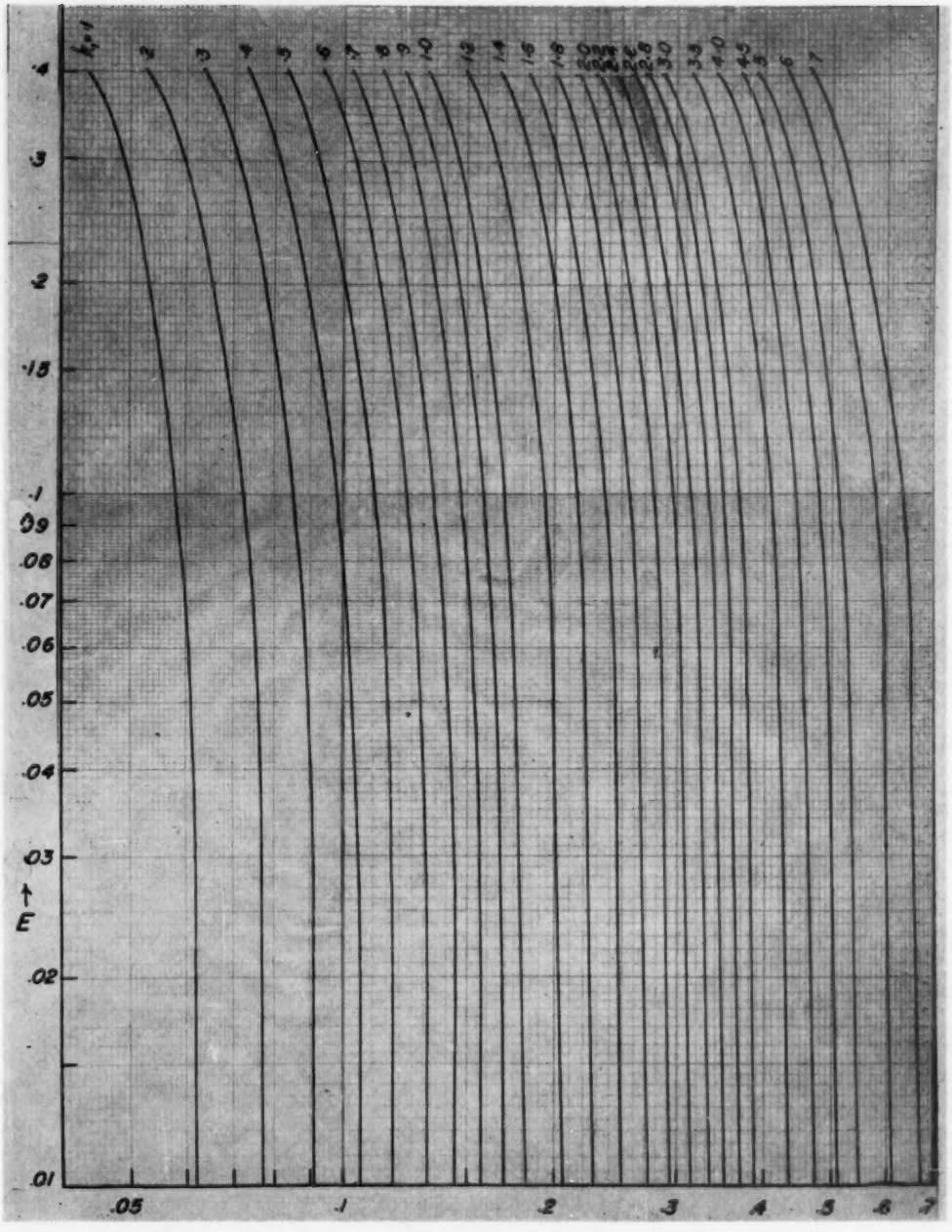

FIG. 3. CHART FOR THYROIDAL RATE k_1 MEASURED AT FOUR HOURS ($t = 240$)

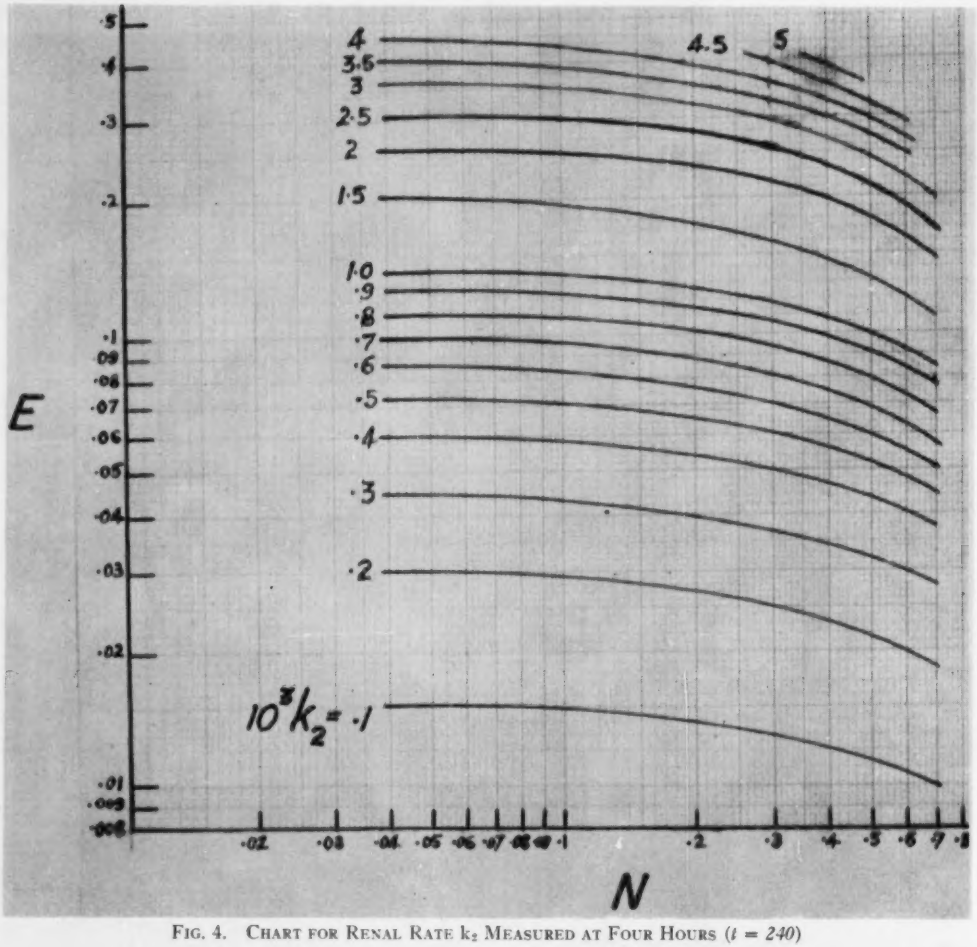

FIG. 4. CHART FOR RENAL RATE k_2 MEASURED AT FOUR HOURS ($t = 240$)

TABLE I

Counting arrangement	Estimated area of neck and torso observed, including penumbral region	Observed $N_0(V_n/V)_2$
Cylindrical Geiger counter in transverse position 15 cm. from skin; open type lead shield similar to arrangement of Werner, Quimby, and Schmidt (2)	550 cm.²	.126
End window Geiger counter, 15 cm. from skin, in cylindrical lead shield of open type.	250 cm.²	.106
Scintillation counter, 47 cm. from skin, short collimator as described earlier (1)	1000 cm.²	.151
Scintillation counter, 47 cm. from skin, long collimator as described earlier (1)	200 cm.²	.073

or, if the initial count is taken over the period 2 to 10 minutes

$$N_0V_n/V = [\phi t N_{2,10} - 8.9\ N] \Big/ \left[\frac{.95\ \phi t (V_n/V)_2}{V_n/V} - 8.9\right] \quad (5)$$

Thus, for example, if t = 60 minutes is the time selected for the second count, the tabu-

TABLE II

Collimator	Number of cases	$N_0(V_n/V)_2$ Mean	$N_0(V_n/V)_2$ Range	Standard deviation of individual readings
Short	17	.146	.074–.272	.047
Long	24	.071	.046–.100	.013

lated values required are found in Table III of the preceding paper as $\phi t = 50.8$ minutes and

$$\frac{V_n/V}{(V_n/V)_2} = 0.84$$ so equation (5) reduces to

$$N_0V_n/V = 1.05\ N_{2,10} - 0.184\ N$$

Similar computations for other times lead to the coefficients listed in Table III.

In the routine case where $N_0(V_n/V)_2 = 0.071$ the derived figures for N_0V_n/V are those in the fourth column of Table III.

CHARTS FOR ROUTINE TESTS

Assuming that routine tests can be timed so that the middle of the period of the uptake observations is at a preselected time, charts are readily constructed to give k_1 and k_2 directly from the observed fractions N and E. Such charts for our long collimator have been derived

TABLE III

Data for finding N_0V_n/V

Time t minutes		Formula for finding from two neck counts N_0V_n/V	Mean N_0V_n/V with long collimator
First observation	Second observation		
2	30	$1.01\ N_2 - 0.144\ N$	.062
2–10 (mean)	30	$1.28\ N_{2,10} - 0.38\ N$	.062
2–10	60	$1.05\ N_{2,10} - 0.184\ N$	.060
2–10	120	$0.91\ N_{2,10} - 0.093\ N$	.056
2–10	240	$0.80\ N_{2,10} - 0.048\ N$	.051
2–10	1,440	$0.56\ N_{2,10} - 0.009\ N$	.037

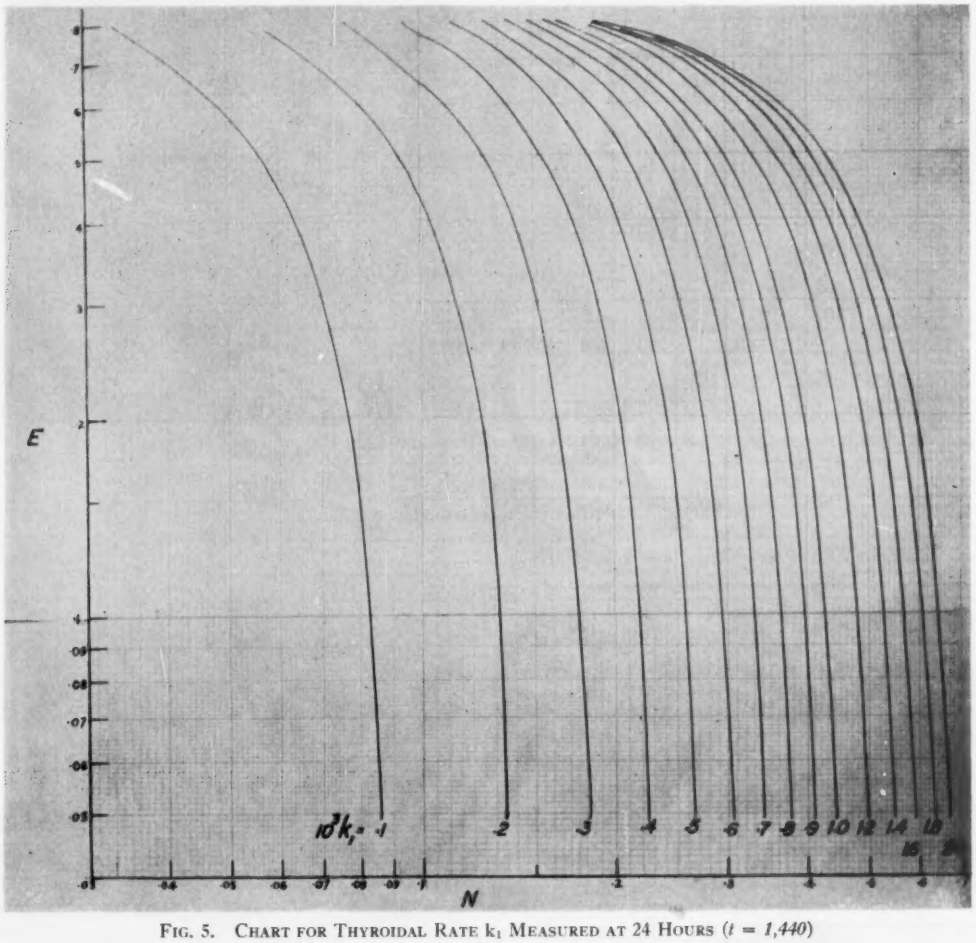

FIG. 5. CHART FOR THYROIDAL RATE k_1 MEASURED AT 24 HOURS ($t = 1{,}440$)

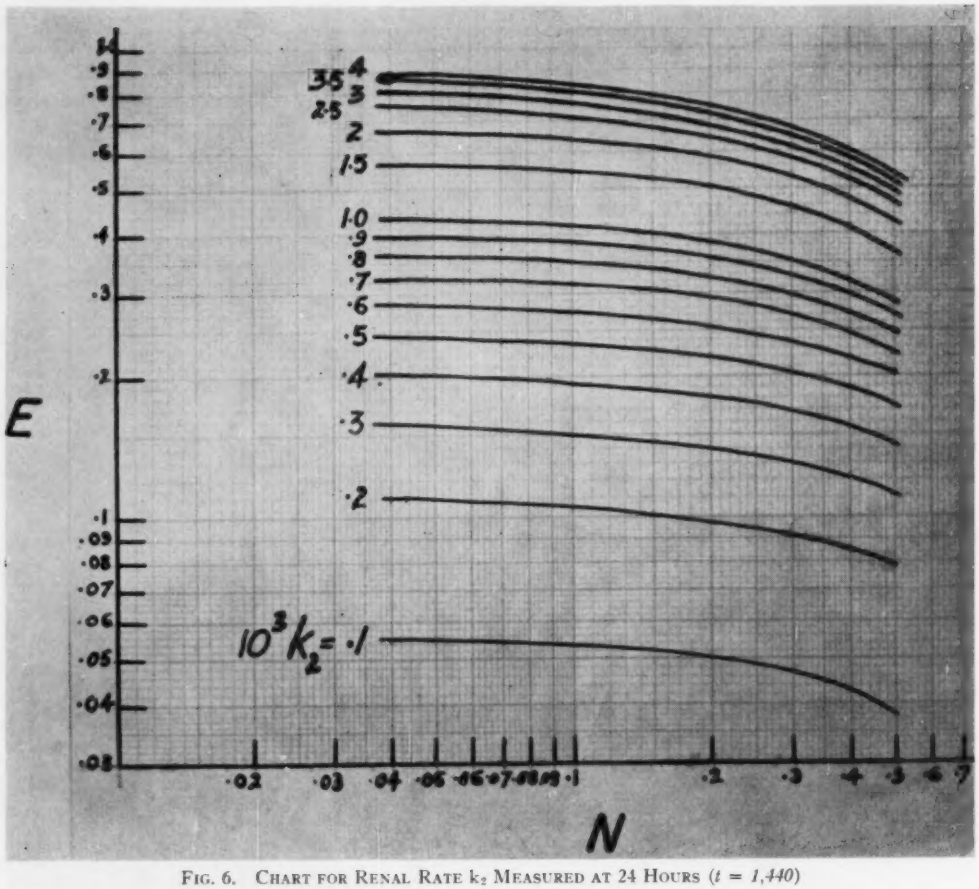

FIG. 6. CHART FOR RENAL RATE k_2 MEASURED AT 24 HOURS ($t = 1,440$)

TABLE IV

Corrections for urine collection time *

Required t†	Actual collection time†	Correction factor for observed E	Required t†	Actual collection time†	Correction factor for observed E
	55	1.15		230	1.06
	60	1.07		235	1.04
60	65	1.00	240	240	1.02
(1 hour)	70	0.94	(4 hours)	245	1.00
	75	0.89		250	0.98
	80	0.84		255	0.96
				260	0.95
	110	1.12		1,400	1.03
	115	1.08		1,415	1.02
120	120	1.04	1,440	1,430	1.01
(2 hours)	125	1.00	(24 hours)	1,445	1.00
	130	0.97		1,460	0.99
	135	0.94		1,475	0.98
	140	0.92		1,490	0.97

* Note that 5 minutes' lag is allowed in urine accumulation, so that urine collected at, for example, 65 minutes, is assumed to be that excreted up to t = 60.

† All times in minutes from injection.

TABLE V

Solution of equation (3) for k at preselected times

$\frac{1-N_0V_n/V}{1-N-E}$	10^4k min^{-1}				
	½ hour t=30	1 hour 60	2 hours 120	4 hours 240	24 hours 1,440
1.00	0.00	0.00	0.00	0.00	0.00
1.05	1.63	0.96	0.56	0.33	0.09
1.10	3.18	1.88	1.10	0.64	0.17
1.15	4.7	2.75	1.61	0.94	0.25
1.20	6.1	3.59	2.10	1.23	0.32
1.25	7.4	4.4	2.56	1.51	0.39
1.30	8.7	5.2	3.02	1.77	0.46
1.35	10.0	5.9	3.45	2.03	0.53
1.40	11.2	6.6	3.87	2.27	0.59
1.45	12.4	7.3	4.3	2.51	0.66
1.50	13.5	8.0	4.7	2.74	0.72
1.55	14.6	8.6	5.0	2.96	0.77
1.60	15.7	9.3	5.4	3.17	0.83
1.65	16.7	9.9	5.8	3.38	0.89
1.70	17.7	10.4	6.1	3.58	0.94
1.75	18.6	11.0	6.4	3.78	0.99
1.80	19.6	11.6	6.8	3.97	1.04
1.85	20.5	12.1	7.1	4.2	1.09
1.90	21.4	12.6	7.4	4.3	1.13
1.95	22.3	13.2	7.7	4.5	1.18
2.00	23.1	13.6	8.0	4.7	1.23
2.1	24.7	14.6	8.5	5.0	1.31
2.2	26.3	15.5	9.1	5.3	1.39
2.3	27.8	16.4	9.6	5.6	1.47
2.4	29.2	17.2	10.1	5.9	1.55
2.5	30.5	18.0	10.5	6.2	1.62
2.6	31.9	18.8	11.0	6.4	1.69
2.7	33.1	19.6	11.4	6.7	1.76
2.8	34.3	20.3	11.8	7.0	1.82
2.9	35.5	21.0	12.2	7.2	1.88
3.0	36.6	21.6	12.6	7.4	1.94
3.1	37.7	22.3	13.0	7.6	2.00
3.2	38.8	22.9	13.4	7.9	2.06
3.3	39.8	23.5	13.7	8.1	2.11
3.4	41	24.1	14.1	8.3	2.16
3.5	42	24.7	14.4	8.5	2.21
3.6	43	25.2	14.7	8.6	2.26
3.7	44	25.8	15.0	8.8	2.31
3.8	44	26.3	15.3	9.0	2.36
3.9	45	26.8	15.6	9.2	2.41
4.0	46	27.3	15.9	9.4	2.45
4.2	48	28.3	16.5	9.7	2.54
4.4	49	29.2	17.0	10.0	2.62
4.6	51	30.1	17.5	10.3	2.70
4.8	52	30.9	18.0	10.6	2.77
5.0	54	31.7	18.5	10.9	2.84
5.5	57	33.6	19.6	11.5	3.01
6.0	60	35.3	20.6	12.1	3.17
6.5	62	36.9	21.5	12.6	3.31
7.0	65	38.3	22.4	13.1	3.44
8.0	69	41	23.9	14.0	3.68
9.0	73	43	25.3	14.8	3.88
10.0	77	45	26.5	15.6	4.1
12	83	49	28.6	16.8	4.4
14	88	52	30.3	17.8	4.7
16	92	55	31.9	18.7	4.9
18	96	57	33.2	19.5	5.1
20	100	59	34.4	20.2	5.3

from equations (*1*), (*2*), and (*3*) using the mean N_0V_n/V from Table III; they are shown in Figures 1 through 6, which give k_1 and k_2 for uptake times of 1, 4, and 24 hours (t = 60, 240 and 1440 minutes).

In using the charts it is assumed that the neck content N is measured over the ten-minute period from t − 5 to t + 5 minutes, and that E is measured with urine *produced* up to t but collected from the bladder at t + 5. If the urine is not collected at exactly this time a correction should be applied from Table IV.

NON-ROUTINE TESTS

In non-routine tests where previous results have been doubtful or where there is reason to suspect that N_0V_n/V may be well away from the expected average value, it is advisable to make initial counts over the neck during the period t = 0 to 10 minutes. This should always be done, if the final count is to be at 30 minutes because the average N_0V_n/V may not be precise enough for this short time of uptake.

Having found N, E, and N_2 or $N_{2,10}$, Table III enables N_0V_n/V to be calculated, ϕt is found from the figures tabulated earlier (1) so that equation (*3*) gives k, equation (*2*) gives k_1 and finally equation (*1*) gives k_3.

To save time if t is kept to one of the selected values (½, 1, 2, 4, or 24 hours), the solution of equation (*3*) for the value of k may be found from Table V. These methods will become clear from the following examples.

EXAMPLES OF CALCULATIONS

Routine case at t = 60 minutes

Let the observed neck fraction, measured with the long collimator from t = 55 to 65 minutes, be N = .235, and the urine fraction collected at t = 73 minutes be .063.

From Table IV the corrected excretion fraction must be $E = .063 \times .91 = .057$.

With these values of N and E, Figures 1 and 2 give

$$k_1 = 4.2 \times 10^{-3} \text{ minute}^{-1}$$
$$k_2 = 1.3 \times 10^{-3} \text{ minute}^{-1}$$

Non-routine case at t = 240 minutes

Let $N_{2,10}$, measured over the period t = 2 to 10 minutes, have an average value of .095; N, observed from t = 235 to 245 minutes, be .106; and E, collected at t = 250 minutes, be .134.

From Table IV the corrected urine value is

$$E = .134 \times .98 = .131$$

From Table III, putting in values

$$N_0V_n/V = .80 \times .095 - .048 \times .106 = .071$$

then

$$\frac{1 - N_0V_n/V}{1 - N - E} = \frac{1 - .071}{1 - .106 - .131} = 1.22$$

and Table V gives $k = 1.34 \times 10^{-3}$ minute^{-1}. From equation (*2*)

$$k_1/k = \frac{.106 - .071 + .009}{.106 - .071 + .131} = .265$$

so that

$$k_1 = .265 \times 1.34 \times 10^{-3} = .35 \times 10^{-3} \text{ minute}^{-1}$$

and from equation (*1*)

$$k_2 = (1.34 - .35) \times 10^{-3} = .99 \times 10^{-3} \text{ minute}^{-1}$$

Non-routine case at t = 190 minutes

Let the measured fractions be $N_{2,10} = .083$, $N = .317$ and the urine collected at $t = 205$, $E = .188$. Because the time of observation, $t = 190$ minutes, is not covered by the charts or Table V, much more calculation is needed in this case.

First E is corrected from the presumed time of excretion (200 minutes) to the time of observation (190 minutes) by multiplying by the ratio of the corresponding ϕt values taken from the preceding paper (1). Thus the corrected value is

$$E = .188 \times 124/129 = .181$$

Then interpolating between the tabulated data of Table III

$$N_0V_n/V = .86\, N_{2,10} - .067\, N = .050$$

Equation (*2*) with these values gives

$$k_1/k = \frac{.317 - .050 + .009}{.317 - .050 + .181} = .616$$

and equation (*3*) gives

$$k = \frac{2.303}{124} \log \frac{1 - .050}{1 - .317 - .181} = 5.1 \times 10^{-3} \text{ minute}^{-1}$$

so that

$$k_1 = .616 \times 5.1 \times 10^{-3} = 3.1 \times 10^{-3} \text{ minute}^{-1}$$

and

$$k_2 = 2.0 \times 10^{-3} \text{ minute}^{-1}$$

DISCUSSION

The method that has been described for calculating the rate constants k_1 and k_2 can be applied regardless of the type of measuring equipment used for uptake observations and urine assay; the charts (Figures 1 through 6), however, are only applicable to collimated counting arrangements with a low average factor $N_0(V_n/V)_2$ of about .07. When other wide-angle apparatus is employed it is necessary to observe the initial counts over the neck (or less preferably over the thigh) to find the correction for extra-thyroidal neck tissue.

Provided that sufficient counts are obtained for good statistical accuracy, and that the collimator is carefully positioned over the patient's gland, a fair degree of accuracy can be expected with the higher values of k_1, if measurements are made one hour after injection. For low-k hypothyroid or cardiac cases, however, there will be little or no rise in the counts over the neck in one hour and we suggest that the four-hour readings should be selected, and in very low cases an additional set of values should be measured at 24 hours also.

The fixing of diagnostic limits for the rate constants k_1 and k_2 depends upon a number of questions distinct from the more physical problems discussed above; in fixing such limits use can be made of some of the information already published by other groups, as will be shown in a later paper describing some of our own clinical results.

In the meantime it can be stated, from the information given in the preceding paper, that the thyroidal rate factor k_1 minute^{-1} is related to the thyroidal clearance rate g ml. per minute, for a 70 Kg. patient, by the relation

$$k_1 = 10^{-3}\, g/16.3$$

This means that, if we take $g = 60$ ml. per minute as the approximate diagnostic borderline between euthyroid and hyperthyroid patients, the corresponding borderline value of k_1 will be around 3.7×10^{-3} minute^{-1}. Thus euthyroid values of k_1 will usually lie below this level, and there will be another not very distinct borderline between hypothyroid and euthyroid groups at about $k_1 = 2 \times 10^{-4}$ minute^{-1}.

SUMMARY

Based on a preceding paper, routine methods are developed for calculating thyroidal uptake and renal excretion rate factors from measured fractions of the dose of radioiodine observed at an early time t after administration.

Charts are included, for selected times t of 1, 4, and 24 hours, giving the rate factors directly from the experimental observations when uptake readings are made with good collimation to keep the extra-thyroidal neck tissue contribution low. Under these conditions such contribution is taken as a mean value for all patients and an uptake study normally requires only one observation over the thyroid gland and one urine assay.

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HEMODYNAMIC EFFECTS OF 1-HYDRAZINOPHTHALAZINE IN PATIENTS WITH ARTERIAL HYPERTENSION [1]

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Although the literature concerning 1-hydrazinophthalazine (Hydralazine) contains many reports of its effects on arterial blood pressure, its relation to various vasomotor reflexes and autonomic blocking agents (1–4), as well as detailed studies of its pharmacological action in animals (5–7), information concerning hemodynamic effects in subjects having essential arterial hypertension is incomplete. The present report concerns various hemodynamic observations in 17 hypertensive patients to whom Hydralazine was administered intravenously.

MATERIAL AND METHODS

This study was done on 17 postabsorptive subjects chosen from the general medical wards. The clinical diagnosis in each case was essential hypertension, although one patient (No. 15) had, in addition, old chronic pyelonephritis involving the lower half of the left kidney. None of these patients was in cardiac decompensation clinically or as measured by the central venous pressure at the time of study. Special effort was made to secure basal conditions by maintaining as much comfort as possible and by explaining the procedure to the patient prior to the day of catheterization and again prior to each step of the procedure. One member of the team tried to establish rapport with each patient during the study by engaging him or her in light conversation about a non-medical subject, preferably one of the subject's choosing.

Cardiac output was determined in the supine position by means of the Fick principle. This was accomplished by placing a cardiac catheter in the pulmonary artery and an indwelling needle in a peripheral artery, usually the femoral. Expired air was collected for three minutes in a Tissot spirometer and analyzed by the Scholander apparatus for O_2 and CO_2 to 0.02 volumes per 100 cc. accuracy. Blood was collected in oiled heparinized syringes during the second minute of the output determination and analyzed for O_2 and CO_2 content by the Van Slyke-Neill method (8). Duplicate analyses of oxygen content were required to check within 0.2 volumes per 100 cc. Direct recordings of blood pressure were made through short flexible plastic tubes connected to Statham strain gauges and through a direct writing Sanborn Poly-Viso. Mean arterial blood pressures were obtained by planimetric integration of the arterial pulse tracing during the first and third minute of the cardiac output determination. Cardiac work and arterial resistances were calculated by the usual formulae (9, 10).

After control determinations, Hydralazine in the dosage indicated in Table I was given through the cardiac catheter into the pulmonary artery. The blood pressure was then observed by continuous inspection of the arterial pressure tracing until it had decreased and stabilized at its new level for at least 10 minutes. An average of 43 minutes elapsed following drug administration before the second cardiac output was determined. During this time some of the subjects had cerebral, some coronary, and some renal hemodynamic studies.

RESULTS

The results are shown in Table I. They are divided into two groups because of the marked yet unpredictable difference in response. Twelve patients, called Group One, experienced mild nasal obstruction, cutaneous facial flushing, and palpitation but no particular discomfort. Five subjects, called Group Two, experienced an adverse reaction characterized by a marked fall in blood pressure, pallor, apprehension, diaphoresis, nausea, and frequently emesis. When this occurred, 100 per cent O_2 was given by mask to produce relief of the patient's discomfort and a rise in blood pressure. In these cases the determination of the second cardiac output was delayed until the blood pressure was stable and symptoms were absent. One patient (No. 16) had a blood pressure fall from a mean of 173 to 24 mm. Hg accompanied by

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[2] American Heart Association Research Fellow.

[3] Wisconsin Heart Association Research Fellow.

TABLE I

Hemodynamic functions before

Patient Age, Sex		Ht.	Wt.	SA	Pulse rate	Syst. MABP	Pulm. MABP	Min. vol. resp.	O_2 cons.	O_2 cons.	Art. O_2 Vol. %	M. ven. O_2 Vol. %	ΔA-V O_2 Vol. %
		Cm.	*Kg.*	*M²*		*mm. Hg*	*mm. Hg*	*L./ min.*	*cc./ min.*	*cc./L. Vent.*			
											Group One—Patients who had moderate		
1 46M	C† D‡	181	84.0	2.05	80 94	161 110	14 14	4.33 8.09	276 339	64 42	17.2 17.6	13.1 14.5	4.1 3.1
2 48F	C D	158	66.5	1.67	78 94	126 105	8 9	4.58 6.27	252 280	55 45	14.1 14.7	10.3 11.6	3.8 3.1
3 35M	C D	182	78.6	2.00	70 92	157 135	19§ 21	6.96 8.12	353 374	42 46	19.9 20.5	15.6 17.3	4.3 3.2
4 49F	C D	152	59.5	1.56	100 100	199 187	14 —	4.74 5.70	208 215	44 38	16.1 15.1	12.8 11.4	3.3 3.7
5 44F	C D	158	45.0	1.42	100 112	160 118	9 10	4.99 6.60	220 235	44 36	15.5 15.2	12.3 11.9	3.2 3.3
6 33F	C D	161	99.0	2.02	87 126	159 115	13 10	7.36 9.55	332 352	45 37	18.3 18.9	14.7 16.3	3.6 2.6
7 57M	C D	179	96.0	2.14	72 73	151 119	16 13	5.91 8.75	292 331	49 38	19.1 20.0	14.8 17.0	4.3 3.0
8 49F	C D	153	58.0	1.56	78 95	153 108	11 9	3.66 4.75	245 242	67 51	19.3 19.8	14.6 16.3	4.7 3.5
9 31M	C D	176	75.5	1.88	94 126	119 117	6 9	9.38 7.27	269 351	29 48	20.3 20.1	15.9 17.0	4.4 3.1
10 60M	C D	173	97.5	2.12	81 93	137 126	9 10	5.23 3.92	210 114	40 36	19.2 19.3	14.5 15.7	4.7 3.6
11 51F	C D	150	53.0	1.45	78 115	157 126	10 6	3.93 4.22	201 186	51 44	17.9 18.1	13.6 13.6	4.3 4.5
12 47M	C D	173	79.3	1.94	78 98	136 128	14 11	5.70 7.60	280 303	49 40	18.2 18.7	13.5 15.3	4.7 3.4
C Ave.					83	151	11	5.56	262	48	17.9	13.8	4.1
D Ave.					102	125	10	6.74	277	42	18.2	14.8	3.3
% Change					+23%	−17%	−9%	+21%	+6%	−13%	+2%	+7%	−20%
P Value					<0.01	<0.01	<0.3	<0.05	<0.3	<0.1	<0.1	<0.01	<0.01
											Group Two—Patients who had marked		
13 49F	C D	160	63.0	1.75	120 98	163 72	11 6	7.71 8.17	281 278	37 34	13.4 13.1	9.7 8.7	3.7 4.4
14 57F	C D	155	61.5	1.60	78 72	163 87	8 7	2.99 4.99	147 188	49 38	19.3 18.2	14.2 14.0	5.1 4.2
15 40M	C D	170	86.0	1.97	91 93	175 72	25 13	11.56 13.74	355 415	31 30	17.8 16.9	11.9 11.4	5.9 5.5
16 50M	C D	171	79.0	1.92	112 125	173 121	20 10	9.65 8.95	339 289	35 32	18.0 16.5	14.2 12.9	3.8 3.6
17 32F	C D	166	57.0	1.63	100 102	168 105	4 7	5.94 7.56	226 196	38 26	19.3 19.0	14.1 14.3	5.2 4.7
C Ave.					100	168	14	7.57	270	38	17.6	12.8	4.8
D Ave.					98	91	9	8.68	273	32	16.7	12.3	4.5
% Change					−2%	−46%	−36%	+15%	+1%	−16%	−5%	−4%	−6%
P Value					<0.8	<0.01	<0.2	<0.1	<0.9	<0.1	<0.02	<0.2	<0.4

* SA M^2—Surface area in square meters.
Syst. MABP—Systemic mean arterial blood pressure in mm. Hg.
Pulm. MABP—Pulmonic mean arterial blood pressure in mm. Hg.
Min. vol. resp. L./min.—Minute volume of air.
O_2 cons. cc./min.—Oxygen consumption, cc. per minute.
O_2 cons. cc./L. Vent.—Oxygen consumption, cc. per litre ventilation.
Art. O_2 Vol. %—Arterial oxygen content in cc. per 100 cc. blood.
M. Ven. O_2 Vol. %—Mixed venous oxygen content in cc. per 100 cc. blood.
ΔA-V O_2 Vol. %—Arterial-mixed venous oxygen difference in cc. per 100 cc. blood.
Art. CO_2 Vol. %—Arterial CO_2 content in cc. per 100 cc. of blood.
M. V. CO_2 Vol. %—Mixed venous CO_2 content in cc. per 100 cc. of blood.
ΔA-V CO_2 Vol. %—Arterial-mixed venous CO_2 difference in cc. per 100 cc. blood.
CO_2 prod. cc./min.—CO_2 expired in cc. per min.
C.I.—Cardiac index in litres per square meter surface area per minute.

TABLE I

and after 1-hydrazinophthalazine *

Art. CO_2 Vol. %	M.V. CO_2 Vol. %	ΔA-V CO_2 Vol. %	CO_2 prod.	C.I.	Tot. syst. resist.	Tot. pulm. resist.	LV work	RV work	Drug dose	Time from drug to CO	C.V.P.	R.Q.
			cc./min.	*L./m²*	*dynes/cm.⁻⁵/sec.*		*Kg. meters/mm.*		*mg./Kg.*	*min.*	*mm. Hg*	
hypotension after hydrazinophthalazine												
46.9	48.9	2.0	165	3.3	1,918	167	14.7	1.3			RA = +3.3	0.60
41.7	43.7	2.0	231	5.3	805	102	16.3	2.1	0.55	56	RA = +0.7	0.68
50.9	54.0	3.1	158	4.0	1,518	97	11.3	0.7			—	0.63
48.0	49.9	1.9	204	5.4	930	79	12.8	1.1	0.25	46	ED = −1.3	0.73
49.8	52.2	2.4	243	4.2	1,485	—	18.0	2.2			ED = +1.5	0.67
44.5	46.3	1.8	287	5.9	923	—	21.5	3.4	0.34	30	ED = 0	0.77
51.9	54.8	2.9	147	4.0	2,523	—	17.1	1.2			RA = 0	0.71
45.4	47.3	1.9	129	3.7	2,571	—	14.8	—	0.32	45	RA = 0	0.60
49.3	51.5	2.2	166	4.8	1,874	105	14.8	0.8			—	0.76
43.4	45.8	2.4	184	5.0	1,341	108	11.2	0.9	0.25	55	ED = 0	0.78
45.3	47.5	2.2	236	4.6	1,379	108	19.9	1.6			RA = −1.9	0.71
39.3	40.7	1.4	259	6.7	678	61	21.2	1.9	0.33	31	RA = −1.8	0.73
43.3	47.7	4.4	228	3.2	1,783	192	13.9	1.5			—	0.78
39.2	41.3	2.1	270	5.2	862	94	17.9	2.0	0.25	29	ED = 0	0.82
49.5	52.2	2.7	165	3.3	2,353	164	10.9	0.8			—	0.67
45.1	48.6	3.5	183	4.4	1,253	105	10.2	0.9	0.25	26	ED = +1.4	0.76
43.0	45.7	2.7	313	3.3	1,554	83	9.9	0.5			—	1.16
39.1	40.7	1.6	221	6.0	823	60	17.9	1.3	0.25	53	RA = −2.2	0.63
39.8	43.9	4.1	164	2.1	2,454	165	8.3	0.6			—	0.78
38.2	40.3	2.1	103	1.9	2,562	197	6.7	0.5	0.20	32	RA = 0	0.73
50.0	53.5	3.5	145	3.2	2,685	160	10.0	0.6			ED = +0.9	0.72
45.9	49.1	3.2	129	2.9	2,427	123	7.1	0.4	0.20	30	ED = +0.9	0.70
45.3	48.1	2.8	200	3.1	1,826	189	11.0	1.1			ED = +3.9	0.71
39.7	41.5	1.8	207	4.6	1,147	96	15.5	1.3	0.25	30	ED = +3.0	0.68
47.1	50.0	2.9	194	3.6	1,963	143	13.3	1.1				0.74
42.5	44.6	2.1	201	4.8	1,360	103	14.4	1.4	0.29	39		0.72
−10%	−10%	−24%	+4%	+33%	−31%	−28%	+8%	+27%				−3%
<0.01	<0.01	<0.01	<0.7	<0.01	<0.01	<0.02	<0.3	<0.05				< 0.7
hypotension after 1-hydrazinophthalazine												
46.4	50.0	3.6	211	4.4	1,713	116	16.9	1.1				0.75
42.9	45.1	2.2	195	3.6	805	81	5.5	0.6	0.35	51	ED = −1	0.70
46.4	49.5	3.1	88	1.8	4,551	246	6.4	0.4			ED = −1	0.60
42.2	44.6	2.4	125	2.8	1,553	129	5.3	0.4	0.25	45	ED = −1.5	0.67
43.5	49.1	5.6	340	3.1	2,326	332	14.3	2.0			ED = 0	0.96
38.5	43.3	4.8	326	3.8	762	132	7.4	1.3	0.30	42	—	0.78
45.1	47.5	2.4	262	4.6	1,551	179	21.0	2.4			ED = +2.4	0.77
45.1	47.7	2.6	242	4.2	1,243	98	13.7	1.1	0.25	102	RA = −0.3	0.84
44.7	48.4	3.7	165	2.7	3,077	81	9.9	0.3			ED = −2	0.73
39.7	42.8	3.1	179	2.6	2,021	131	6.0	0.4	0.25	32	ED = −2	0.92
45.2	48.9	3.7	213	3.3	2,644	191	13.7	1.2				0.76
41.7	44.7	3.0	213	3.4	1,277	114	7.6	0.8	0.28	54		0.78
−8%	−9%	−19%	0	+3%	−52%	−40%	−45%	−33%				+3%
<0.02	<0.02	<0.05	0	<0.9	<0.05	<0.2	<0.02	<0.2				—

* Tot. syst. resist.—Total systemic arterial resistance in dynes per cm.⁻⁵/sec.
Tot. pulm. resist.—Total pulmonary arterial resistance in dynes per cm.⁻⁵/sec.
LV work—Left ventricular work in Kg. meters per minute.
RV work—Right ventricular work in Kg. meters per minute.
Time from drug to CO—Time in minutes between the administration of the drug and the determination of the second cardiac output.
C.V.P.—Central venous pressure measured either as right ventricular end diastolic pressure (ED) or right atrial mean pressure (RA) in mm. Hg.
R.Q.—Respiratory quotient for total body.

† Control.
‡ After Hydralazine.
§ Right ventricular systolic mean.

nodal bradycardia. He was given O_2 by mask, then methoxamine 2 mg. intravenously, followed by 12 mg. intramuscularly, to maintain his pressure high enough for the second output. The data derived from this study would have been omitted from the report except that his response is similar to the rest of those in Group Two. The average dose of Hydralazine was the same in both groups.

The cardiac rate in Group One increased from a mean in the control period of 83 beats per minute to a mean of 102 beats per minute ($p < 0.01$) after the intravenous administration of Hydralazine. In Group Two, who had marked hypotension, no increase in pulse rate occurred, and in two cases (No. 15 and No. 16) there was nodal rhythm with bradycardia during the period of greatest hypotension.

Systemic arterial blood pressure consistently fell. The average fall of the mean pressure in Group One was 26 mm. Hg ($p < 0.01$), and in Group Two 77 mm. Hg ($p < 0.01$). In Group Two the average maximum fall in blood pressure was 108 mm. Hg and was usually transient. Effort was made to prevent such falls by decreasing the Hydralazine dose gradually during the series but they occurred unpredictably. The decrease in the pulmonary arterial pressure was not statistically significant in either group. Calculation of cardiac output indicated that there was an increase of 33 per cent ($p < 0.01$) in Group One and no change in Group Two. Stroke volume rose slightly but not significantly in both groups. Peripheral arterial resistance was reduced by 31 per cent ($p < 0.01$) in Group One and 52 per cent in Group Two ($p < 0.05$), whereas total pulmonary resistance fell by 28 per cent ($p < 0.02$) and 40 per cent ($p < 0.2$), respectively, in the two groups. With the concomitant fall in peripheral arterial blood pressure and the change in cardiac output, cardiac work against pressure fluctuated unpredictably. The left ventricular work fell in Group Two (-45 per cent, $p < 0.02$) and right ventricular work rose in Group One ($p < 0.05$).

In Group One O_2 consumption and arterial O_2 level were not significantly changed by Hydralazine. The increased cardiac output produced a decrease in arteriovenous O_2 difference ($p < 0.01$) because of the increase in the mean mixed venous O_2 content of 1.0 volume per 100 cc. By contrast, in Group Two there was a fall in arterial O_2 content ($p < 0.02$). Here the variability of mixed venous O_2 was such that no statistically significant change occurred in the arteriovenous O_2 difference. Oxygen consumption remained the same.

In 10 patients of Group One the minute volume of respiration increased after the administration of Hydralazine, the average increase for the entire group being 21 per cent ($p < 0.05$). As would be expected in these circumstances, the arterial CO_2, mixed venous CO_2 and arterio-mixed venous CO_2 difference all decreased ($p < 0.01$). In Group Two the arterial and venous blood CO_2 content changes were similar but less significant, probably because of the smaller number of patients. Constant CO_2 production and unchanged respiratory quotients of both groups attested to the attainment of a reasonably steady state by the time of the second hemodynamic determinations.

DISCUSSION

A previous report of cardiac output after administration of Hydralazine was made by Wilkinson, Backman, and Hecht (11) in a total of six patients. They found a markedly greater increase in the cardiac output in four normotensive patients (average + 112 per cent) than in two hypertensive patients (average + 19 per cent). Further data are reported by Assali, Kaplan, Oighenstein, and Suyemoto (12) who by the ballistocardiographic method demonstrated an increase in cardiac output and a decrease in peripheral resistance after Hydralazine administration to pregnant normotensive, toxemic and hypertensive subjects. A similar pattern was found by the Fick principle in their three patients with toxemia. Moyer (13) by the pulse contour method, and Freis, Rose, Finnerty, and Partenope (14) by the Fick principle demonstrated an increase in cardiac output. Our observations in general confirm and extend these earlier reports.

The data from Group One suggest that the primary change after administration of Hydralazine to these patients with hypertension is a decrease in the vascular resistance both in the systemic cir-

culation (− 31 per cent) and in the lung (− 28 per cent). Accompanying this decrease in vascular resistance there is a fall in systemic arterial pressure (− 17 per cent) and a rise in cardiac output (+ 33 per cent). In spite of the increase in cardiac output, calculated left ventricular pressure work is not increased and the O_2 consumption remains essentially the same. It seems clear that the vasodilatation produced by this drug is quite general since it is found in cerebral (15, 16), hepatic (7, 14), renal (11, 17), and coronary vascular beds (18) as well as in the systemic and pulmonary vascular beds as a whole as shown in this study. However, the degree of vasodilatation is not the same in all areas (11, 18). Skin temperature was not found to increase generally by Wilkinson, Backman, and Hecht but did occur locally after direct intra-arterial administration (11). It was shown to rise in local areas after intravenous administration in pregnant patients by Assali, Kaplan, Oighenstein, and Suyemoto (12).

The changes produced in Group Two were complicated by the precipitation of marked hypotension and the resulting adjustments which occur secondary to such a hemodynamic condition. It seems probable that this marked hypotensive effect was due to failure of venous return because of peripheral and splanchnic pooling of blood. This cannot be stated with certainty, since the central venous pressure was measured at the beginning and end of the procedure, not during the most hypotensive phase. The slight fall in arterial O_2 content appears related to the marked hypotensive phase.

A mild degree of hyperventilation occurred in both groups as shown by the increase in minute volume of respiration, and further manifested by the fall in arterial and venous CO_2 contents. Although pH determinations were not done in this study, arterial pH as determined by Hafkenschiel and his associates (15) showed a slight rise after administration of Hydralazine. The reason for this hyperventilation is not known, but the fact that it was less in the Group Two patients, who were most apprehensive and uncomfortable, suggests that it was not psychogenic.

During the study and in retrospect since its completion an attempt has been made to determine what factor or factors accounted for the different response of Group One and Group Two. Statistical testing indicates that Group Two patients had, on the average, higher mean arterial blood pressure ($p < 0.02$), lower stroke index ($p < 0.05$), and lower O_2 consumption per liter ventilation ($p < 0.05$) than Group One patients. None of the other factors which we measured could be shown statistically to be related to the difference in response. It is interesting to speculate that the response to 1-hydrazinophthalazine may be related to cardiac reserve. The increase in cardiac output of normal patients (Wilkinson, Backman, and Hecht) was 112 per cent, our Group One hypertensive subjects had only a 33 per cent increase and the Group Two hypertensive subjects, with an even higher arterial blood pressure, showed no significant change. Other patients in Group One who did not experience adverse symptoms but who had severe hypertension, *e.g.*, No. 4, or a very low initial cardiac output, as No. 10, also had no increase in cardiac output after the drug was given. This suggests that there is a continuous spectrum of response from the patient who becomes flushed and warm, develops tachycardia, and increases his cardiac output and left ventricular work to the patient who develops marked hypotension, pallor, sweating, apprehension, bradycardia with nodal rhythm, and whose cardiac output and cardiac work fall.

CONCLUSIONS

1. Administration of 1-hydrazinophthalazine intravenously to 17 subjects with arterial hypertension produced a decrease in peripheral and pulmonary vascular resistance with a decrease in systemic arterial blood pressure accompanied generally by increased cardiac output. However, when severe hypotension occurred, cardiac output remained the same or fell.

2. Left ventricular work fell when marked hypotension occurred, but otherwise was unchanged.

3. Hyperventilation occurred after Hydralazine administration in 14 of 17 patients with a fall in arterial and mixed venous CO_2 and a decrease in arteriovenous CO_2 difference.

4. No significant change occurred in O_2 consumption, total CO_2 production, or respiratory quotient.

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CEREBRAL HEMODYNAMICS IN PATIENTS WITH HEART FAILURE ASSOCIATED WITH HYPERTENSION AND THE RESPONSE TO TREATMENT[1]

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Cerebral blood flow and cerebral oxygen consumption are not altered in patients with uncomplicated hypertension (1). Scheinberg observed that in a mixed group of patients who had congestive heart failure, the cerebral blood flow was depressed (2). This suggests altered cerebral hemodynamics either as a primary abnormality or as a result of disturbances in the cerebral circulatory dynamics secondary to the cardiac failure. Novack, Goluboff, Bortin, Soffe, and Shenkin (3) studying the same problem concluded that the cerebral blood flow is not depressed to any greater extent in patients with heart failure than it would be in a similar group of patients of the same age group who were not in heart failure. Since cerebral blood flow and cerebral oxygen consumption are relatively normal (1, 4) in patients with essential hypertension which is uncomplicated by the co-existence of heart failure, it seemed worth making observations on cerebral hemodynamics in a group of patients with hypertension who had developed heart failure. Since all but three of these patients were under the age of sixty years, if a significant reduction in cerebral blood flow were found, it could then be considered to be a result of the heart failure rather than secondary to cerebral arteriosclerosis. In addition, observations were made on the cerebral hemodynamic response when twelve patients were given maximum therapy for heart failure. If cerebral blood flow and oxygen consumption were depressed because of heart failure, one might well have expected these functions to improve following treatment.

METHODS AND MATERIALS

Observations on cerebral hemodynamics and cerebral oxygen metabolism were made on twenty patients with hypertension complicated by heart failure and six patients with heart failure due to other causes. Similar observations were made from four days to one month later on fourteen of these patients after they had received maximum benefit from treatment for heart failure. Although all but one patient showed marked subjective and objective improvement of symptoms and signs following therapy, the circulation time and venous pressure had not returned to normal in all of them. Twelve of the fourteen patients who were studied before and after long-term therapy had hypertension. Treatment included digitalis, diuretic therapy, low-salt diet, and bed rest when indicated. Observations were also made before and one-half hour to two hours after rapid digitalization in five patients. Cerebral blood flow was measured by the nitrous oxide method (5). The jugular venous pressure was measured with a spinal fluid manometer using the level of the venopuncture site (jugular bulb) as the reference point. The mean arterial pressure (MABP) was measured by direct arterial manometry using the femoral artery. The methods and analytical procedures used have been described previously (4, 6). The partial pressure of carbon dioxide (pCO_2) was determined with the aid of a Van Slyke Nomogram (7). The cerebral oxygen consumption ($CMRO_2$) was determined by multiplying cerebral blood flow by the arteriovenous oxygen difference. The cerebrovascular resistance (CVR) as presented in the current study is a simple ratio of effective mean blood pressure divided by cerebral blood flow per 100 grams of brain per minute.

At the time of the initial study all of the patients had peripheral edema and some dyspnea associated with varying degrees of heart failure. Because of technical difficulties in carrying out the procedure, patients with very severe dyspnea were excluded from the study. The severity of disease in the twenty patients with hypertension varied from mild essential hypertension to severe malignant hypertension. In one patient (No. 6) the hypertension was associated with chronic glomerular nephritis. All of the studies were done with the patients in the supine position, although in several patients it was necessary to flex the neck and thorax because of moderate orthopnea. When this became necessary in order to complete the initial observations, the post treatment observations were made with the patient in the same position. Studies were also completed on six patients with heart failure due to causes other than hypertension. In addition to the twenty-six patients herewith reported, studies were attempted on five other patients, but the

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TABLE I

Clinical status of patients with heart failure and the effect of treatment *

Patient	Sex	Age	Clinical diagnosis	Degree of heart failure	Hematocrit (%) C	Hematocrit (%) D	Circulation time† (sec.) C	Circulation time† (sec.) D	Venous pressure mm. water C	Venous pressure mm. water D	Respiratory rate (per min.) C	Respiratory rate (per min.) D	Pulse rate (per min.) C	Pulse rate (per min.) D	Mean blood pressure‡ mm. Hg C	Mean blood pressure‡ mm. Hg D	Date of study C	Date of study D
a. Patients with hypertension on whom studies were done before and after treatment for heart failure																		
1.§	F	59	HCVD	2+	36	35	35	20	260‡‡	160‡‡	31	14	74	70	133	109	6-8	6-12
2.§¶	M	56	HCVD	3+	36	36	20	15	241‡‡	132‡‡	34	21	110	94	155	130	5-15	5-29
3.	M	57	HCVD, SHD	2+	42	41	35	25	111	90	42	44	100	78	150	127	6-29	7-6
4.§	F	51	HCVD	3+	35	38	28	15	103	62	17	19	110	96	160	135	9-5	10-2
5.§	F	56	HCVD	3+	42	40	50	22	180	100	48	18	86	78	172	127	9-12	9-25
6.§††**	M	46	HCVD, CGN	2+	35	37	37	21	240‡‡	140‡‡	20	26	80	90	157	180	9-25	10-3
7.	F	46	HCVD	1+	28	33	28	20	230‡‡	130‡‡	20	20	86	72	150	133	6-10	6-30
8.	M	34	HCVD	1+	49	38	—	—	130	100	26	30	96	88	167	174	10-7	10-11
9.	M	65	HCVD	4+	42	49	—	—	250	196	29	20	98	62	152	140	4-17	5-1
10.	M	59	HCVD	3+	39	48	17	13	112	82	56	17	127	76	182	143	4-24	5-29
11.	M	50	HCVD	3+	38	36	—	—	—	191	24	—	90	92	173	135	8-10	8-14
12.	M	40	HCVD	2+	42	40	50	19	210	61	20	14	92	75	147	127	7-17	7-24
Mean		52			39	39	33	19	188	120	31	22	96	81	158	138		
% of control						100		58		64		71		84		87		
P <						0.50		0.01		—		0.10		0.01		0.01		
b. Patients with hypertension on whom post treatment studies were not done																		
13.	F	48	HCVD	1+	34		—		211		15		78		145			
14.	F	46	HCVD	1+	47		17		245		20		88		150			
15.	M	34	HCVD	1+	43		20		40		20		96		195			
16.	M	59	HCVD	1+	47		23		184		31		124		118			
17.	F	44	HCVD	4+	42		—		—		32		110		153			
18.	F	63	HCVD	3+	26		30		146		22		95		143			
19.	M	30	HCVD	3+	51		31		146		33		120		168			
20.	F	65	HCVD	3+	42		42		256		24		114		153			
Mean		49			42		27		175		25		103		153			
Grand Mean ±S.E.‖‖		50			40±1.5		31±2.8		183		28±2.4		99±3.4		156±3.8			
c. Patients in heart failure not associated with hypertension																		
21.	M	34	Etiology?	4+	38		34	24	170‡‡	110‡‡	26	20	100	92	93	105	3-6	3-20
22.	M	46	RHD, AI, MS	3+	55		55	25	200‡‡	140‡‡	22	16	104	88	58	58	4-27	5-22
23.	M	58	SHD, AI	3+	50		35	—	170‡‡	—	28	—	96		93			
24.	M	24	RHD, AI	1+	39		14		80		20		124		80			
25.	M	41	RHD, MS	2+	52		18		30		20		72		98			
26.	M	61	SHD, AI	2+	30		28		140		36		108		95			
Mean		44			44		31	25	132	125	25	18	101	90	86	82		
S.E.‖‖					4.0		6.0				2.5		7.0		6.2			

* C—Control.
D—After treatment of heart failure with digitalis, mercurials, etc.
HCVD—Hypertensive cardiovascular disease.
RHD—Rheumatic heart disease.
AI—Aortic insufficiency.
MS—Mitral stenosis.
SHD—Syphilitic heart disease.
CGN—Chronic glomerular nephritis.
† Decholin@ circulation time in seconds.
‡ Mean blood pressure—direct arterial manometry.
§ Received NH_4Cl before and during studies.
‖ Azotemia—Patient 2—BUN = 34 mg. per cent 5/15 and 16 mg. per cent 5/29.
Patient 4—BUN = 27 mg. per cent during control study, 14 mg. per cent after treatment.
¶ Poor results with treatment.
** Hemoglobin = 10 gm.
†† Confused.
‡‡ Antecubital venous pressur ; all others are jugular bulb pressure.

‖‖ ±S.E. – Standard Error = $\sqrt{\frac{SX^2}{N(N-1)}}$. Standard error for patients 1 to 20.

‖ ‖ Standard error for patients 21 to 26.

procedure had to be discontinued because of dyspnea and discomfort when the mask was applied. The blood urea nitrogen was elevated in two patients (Numbers 2 and 4) apparently as a result of the heart failure, since there was no evidence of primary renal disease in these patients. The venous pressure was moderately elevated in fourteen of the twenty-four patients so studied, and the circulation time was increased in twenty of the twenty-one on whom this observation was made. The average age of the patients with hypertension was fifty years. The heart failure responded well to therapy in all but one of the patients (Number 2) who were treated. The two patients who were receiving ammonium chloride prior to therapy continued to receive this drug during the treatment period. The remainder of the patients did not receive acidifying salts before or during the study. About half of the patients had not previously received treatment for heart failure and the remainder had failed

to follow an adequate treatment program prior to these studies.

RESULTS

The clinical status of the patients and the mean blood pressure are presented in Table I. The cerebral hemodynamics and the response to treatment are presented in Table II.

Although the cerebral blood flow seemed to be lower than normal in a few instances, there was no significant difference in cerebral blood flow for the group of patients with heart failure associated with hypertension (51 ± 2.9 ml. per min.) than in normal subjects (5) or patients with hypertension (1, 4) who did not have heart failure. In

TABLE II

Cerebral hemodynamics and cerebral blood oxygen and carbon dioxide in patients with heart failure and the effect of treatment *

Patient	Arterial CO_2 *volume %* C	D	Venous CO_2 *volume %* C	D	Arterial P CO_2 *mm. Hg* C	D	Arterial O_2 *volume %* C	D	Venous O_2 *volume %* C	D	A-V O_2 *volume %* C	D	Cerebral blood flow *ml./min.* C	D	Cerebral O_2 uptake° *ml./min.* C	D	CVR C	D
a. Patients with hypertension on whom studies were done before and after treatment of heart failure																		
1.†	29.5	31.9	35.0	38.5	—	—	17.6	16.6	10.8	9.7	6.8	6.9	37	50	2.5	3.5	3.6	2.2
2.‡‖	19.2	21.7	25.8	29.6	32	36	11.9	12.7	5.4	4.3	6.5	8.4	55	41	3.6	3.4	2.8	3.2
3.	37.4	37.0	45.9	44.9	—	—	15.6	17.8	7.4	9.2	8.2	8.6	32	43	2.6	3.7	4.7	3.0
4.‡	26.0	43.6	32.4	49.9	36	38	14.6	14.1	8.2	7.6	6.4	6.5	48	57	3.1	3.7	3.3	2.4
5.†	24.9	51.0	33.7	61.0	28	44	16.6	18.1	9.4	9.3	7.2	8.8	59	45	4.2	4.0	2.9	2.8
6.§¶	51.6	55.0	55.0	60.0	49	47	8.8	8.7	5.0	4.5	3.8	4.2	50	49	1.9	2.1	3.1	3.7
7.	39.4	37.2	45.3	43.5	36	33	16.0	15.9	10.8	9.9	5.2	6.0	74	70	3.8	4.2	2.0	1.9
8.	48.7	45.1	52.1	51.8	38	40	16.2	12.7	11.2	8.1	5.0	4.6	64	55	3.2	2.5	2.6	3.2
9.	39.8	40.8	47.5	46.1	32	38	16.8	20.7	10.3	11.8	6.5	8.9	53	36	3.4	3.2	2.9	3.9
10.	34.8	42.9	41.5	52.3	29	37	10.6	16.5	5.8	7.9	4.8	8.6	35	58	1.7	5.0	5.2	2.5
11.	29.0	31.4	35.8	37.4	—	—	15.1	16.8	8.5	8.3	6.6	8.5	63	48	4.2	4.1	2.7	2.8
12.	42.7	39.8	49.6	45.6	32	31	12.0	13.4	5.7	9.0	6.3	4.4	49	46	3.1	2.0	3.0	2.8
Mean	35.3	39.8	41.6	46.7	35	38	14.3	15.3	8.2	8.3	6.1	7.0	52	50	3.1	3.5	3.2	2.9
% of control		113		112		109		107		101		115		96		113		91
P <		0.30		0.10		0.10		0.20		0.50		0.10		0.50		0.40		0.30
b. Patients with hypertension on whom post-treatment studies were not done																		
13.	29.6		33.2		31		16.2		7.2		9.0		43		3.9		3.4	
14.	38.4		45.3		38		16.0		10.8		5.2		56		2.9		2.7	
15.	44.6		51.8		31		14.7		9.1		5.6		38		2.1		5.1	
16.	26.4		31.9		—		16.8		11.8		5.0		65		3.3		1.8	
17.	32.1		36.5		—		15.6		5.8		9.8		47		4.6		3.3	
18.	41.0		45.0		38		13.5		8.0		5.5		40		2.2		3.6	
19.	34.6		42.3		30		18.8		8.7		10.1		32		3.2		5.3	
20.	39.8		49.5		32		16.8		10.3		6.5		73		4.7		2.1	
Mean	35.8		41.9		33		16.1		9.0		7.1		49		3.4		3.4	
Grand Mean ±S.E.**	35.5 ± 1.92		41.8 ± 2.65		34 ± 1.0		15.0 ± 0.64		8.5 ± 0.48		6.5 ± 0.38		51 ± 2.9		3.2 ± 0.20		3.3 ± 0.23	
c. Patients in heart failure not associated with hypertension																		
21.	37.8	48.4	46.4	55.0	—		18.5	14.7	6.2	7.5	12.3	7.2	22	57	2.7	4.1	4.2	1.8
22.	29.5	31.9	35.0	38.5	—		17.6	16.6	10.8	9.7	6.8	6.9	37	50	2.5	3.5	1.6	1.2
23.	25.0	—	33.2	—	—		15.2	—	6.5	—	8.7	—	31	—	2.7	—	3.0	—
24.	41.6	—	48.5	—	37		15.8	—	9.1	—	6.7	—	38	—	2.5	—	2.1	—
25.	40.8	—	46.0	—	39		18.4	—	12.2	—	6.2	—	41	—	2.5	—	2.4	—
26.	20.7	—	28.0	—	—		12.7	—	8.0	—	4.7	—	46	—	2.2	—	2.1	—
Mean	32.6	40.2	39.5	46.8	38		16.4	15.7	8.8	8.6	7.6	7.1	36	54	2.5	3.8	2.6	1.5
Std. Error	3.30		3.40		1.0		0.92		0.97		1.08		3.4		0.24		0.37	

* C—Control.
D—After treatment for heart failure.
CVR—Cerebrovascular resistance = $\frac{\text{Mean Blood Pressure}}{\text{Cerebral Blood Flow}}$.
°—Cerebral blood flow in ml. per 100 grams of brain times Arterial-Venous Oxygen difference.
P CO_2—Partial pressure of carbon dioxide in mm. Hg.
A-V O_2—Arterial-Venous O_2.
† Received NH_4Cl before and during studies.
‡ Azotemia—Patient 2—BUN = 34 mg. per cent 5/15 and 16 mg. per cent 5/29.
Patient 4—BUN = 27 mg. per cent during control study, 14 mg. per cent after treatment.
§ Hemoglobin = 10 gm.
Poor results with treatment.
¶ Mentally confused.

** ±Standard Error − Standard Error = $\sqrt{\frac{SX^2}{N(N-1)}}$. This is the standard error for patients 1 to 20.
S.E.—Standard Error.

four of the patients (Numbers 3, 10, 19, and 21) in whom the cerebral blood flow appeared to be depressed, dyspnea was marked. Under these circumstances one might anticipate a low pCO_2 due to hyperventilation which in itself could cause a reduction in cerebral blood flow (8) rather than on the basis of a reduction in cardiac output (2). However, a number of patients with marked dyspnea showed entirely normal cerebral blood flow and cerebral oxygen consumption. As in patients with hypertension (1) without heart failure, the cerebrovascular resistance was increased in these patients (3.3 ± 0.2). Cardiac failure did not appear to alter these dynamics. Both patients (Numbers 2 and 4) with azotemia had normal cerebral blood flow and cerebral oxygen consumption.

Treatment for heart failure in twelve of the patients with hypertension resulted in a significant reduction in mean blood pressure ($p < 0.01$). However, cerebral blood flow did not change significantly. It increased in four patients, decreased in five, and in three it was approximately the same after treatment as it was before therapy. The averages for the group before and after treatment were nearly identical (Table II). There was a slight increase in cerebral oxygen uptake for the group, but this response was neither great enough nor consistent enough to be statistically significant. The increase in arteriovenous oxygen difference was due primarily to increased oxygen content in the arterial blood, presumably a result of improved pulmonary oxygen exchange. There was an increase in both arterial ($p < 0.30$) and venous ($p < 0.10$) blood CO_2 content associated with an increase in partial pressure (pCO_2) ($p < 0.10$). These alterations were not statistically significant when analyzed by paired differences[2] but the changes were fairly consistent, and were probably a result of the relief of the hyperventilation associated with the pulmonary congestion. There was very little alteration in the arteriovenous blood CO_2 content.

Rapid digitalization with 0.68 to 1.4 mgm. of Strophanthin K given intravenously had no effect on cerebral blood flow or on cerebral oxygen uptake 30 to 120 minutes after administration of the drug in the five patients studied. For example, one patient with a low cerebral blood flow of 34 ml. per minute and 37 ml. per minute during successive control periods taken 30 minutes apart showed a cerebral blood flow of 30 ml. per minute one-half hour after Strophanthin K administration and 38 ml. per minute one and one-half hours later. The mean value for cerebral blood flow in five patients was 44 ml. per minute before rapid digitalization and 45 ml. per minute after it. The cerebral blood flow in six patients with heart failure due to diseases other than hypertension (syphilis, chronic rheumatic heart disease, and etiology unknown in one instance) was reduced significantly, although the age range was approximately the same as in the twenty patients with heart failure associated with hypertension (Table II).

[2] $t = \bar{x}\sqrt{\frac{N(N-1)}{\Sigma x^2}}$

Statistics completed by Dr. R. A. Seibert.

COMMENTS

The current observations appear to support the contention that cerebral blood flow is not depressed in patients with heart failure (3) when the heart failure is due to hypertension. It appears that heart failure *per se* did not depress cerebral blood flow or cerebral oxygen consumption, since the average cerebral blood flow in this group of patients with heart failure was about the same as in normal subjects (5, 9) as well as patients with hypertension (1, 4). As Novack, Goluboff, Bortin, Soffe, and Shenkin (3) have pointed out, arteriosclerosis itself is frequently associated with a reduction in cerebral blood flow. When these patients develop heart failure cerebral blood flow will accordingly be lower than in normal young subjects. The fact that adequate and prolonged treatment with improvement of the heart failure in our patients did not increase cerebral blood flow or cerebral oxygen consumption, is further evidence that the cerebral blood flow was probably not depressed due to heart failure in the first place. Likewise, rapid digitalization with Strophanthin K did not alter cerebral hemodynamics. The patients with low values for cerebral blood flow before treatment were usually dyspneic and also exhibited low pCO_2 values, apparently a result of the hyperventilation (8). Patients with prerenal azotemia did not show a decrease in cerebral blood flow despite the low

pCO_2 and CO_2 content and acidosis exhibited by them. Similarly, the cerebral oxygen consumption was not altered.

Further observations are indicated on patients with cardiac failure due to causes other than hypertension before dogmatic and inclusive deductions can be made about congestive heart failure, irrespective of the etiology of the disease. The current observations on six patients (Numbers 21 to 26) suggest that cerebral blood flow is reduced in patients with rheumatic and syphilitic heart disease associated with heart failure. However, the reduction in cerebral blood flow in these cases may be due to the mechanics of the underlying cardiac disease (aortic insufficiency or mitral stenosis) resulting in a greater depression in cardiac output and may not be a result of the accompanying congestive heart failure.

In order to further elucidate this problem, cerebral blood flow determinations have been done before and after tourniquets were applied around the necks of three normal subjects and four patients with heart failure who had received adequate therapy. The tourniquet pressure was increased until the venous pressure in the jugular bulb was increased to 120 to 300 mm. Hg. Cerebral blood flow was not altered by this procedure (10). This indicates that cerebral blood flow is not reduced by increases in venous pressure within the limits present in patients with congestive heart failure. However, it is possible that cerebral hemodynamics and cerebral oxygen consumption may be altered in patients with severe pulmonary congestion associated with dyspnea due to severe heart failure. Under these conditions, it is impossible to do accurate cerebral blood flow determinations using the nitrous oxide technique.

SUMMARY

1. Observations have been made on cerebral hemodynamics and cerebral oxygen consumption in twenty patients with hypertension associated with heart failure. Cerebral blood flow and cerebral oxygen consumption were not altered in these patients.

2. Treatment for heart failure did not alter cerebral blood flow or cerebral oxygen consumption in patients with hypertension and associated cardiac failure.

3. Cerebral blood flow was depressed in six patients who had heart failure associated with aortic insufficiency or mitral stenosis.

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TRANSAMINASE ACTIVITY IN HUMAN BLOOD

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Enzymatic transamination consists of the enzyme catalyzed reversible transfer of the alpha amino nitrogen of an amino acid to an alpha-keto acid with the synthesis of a second amino acid and a second alpha-keto acid. Enzymes catalyzing different transamination reactions are found widely distributed in animal tissues and have been shown to change in activity in some tissues during disease (1–3). These observations prompted the present study to determine if transaminase activity could be demonstrated in human serum and blood cellular elements and, if so, to study any variations in activity of this enzyme in the blood of normal and diseased man.

METHODS AND MATERIALS

The two transaminases found most active in animal tissues are:

1. *"Glutamic-oxalacetic transaminase"*
   Aspartate + alpha-keto glutarate ⇌ glutamate + oxalacetate
2. *"Glutamic-pyruvic transaminase"*
   Alanine + alpha-keto glutarate ⇌ glutamate + pyruvate

When aspartate or alanine are incubated with alpha-keto glutarate and a source of enzyme, the rate of production of glutamate may be taken as a measure of transaminase activity. The amount of glutamate produced after a given incubation period under standardized conditions was measured by quantitative paper chromatographic analysis (4).

One-tenth molar solutions of l-aspartate, l-alanine, and alpha-keto glutarate were prepared in 0.06 M phosphate buffer and the pH of the solutions adjusted to pH 7.6. For serum transaminase determinations, 0.5 ml. of clear, non-hemolyzed serum, 1.5 ml. of 0.06 M phosphate buffer, pH 7.6, and 0.5 ml. of either the alanine or aspartate solutions were incubated for ten minutes at 37°C. At this time, 0.5 ml. of the alpha-keto glutarate solution was added and the incubation continued for 18 hours. For whole blood hemolysate transaminase determinations, equal volumes of blood and distilled water were shaken together for ten minutes, 1.0 ml. of the hemolysate was added to 1.0 ml. of the phosphate buffer, and the substrates added as above. The time of incubation of the hemolysate substrate mixture was three hours.

At the end of the incubation period, proteins were separated by adding 7.0 ml. of absolute ethyl alcohol, centrifuging for ten minutes, and washing the precipitate with 5 ml. of 70 per cent ethanol. The supernatant was evaporated to dryness over a water bath and the residue dissolved in 1.0 ml. of 0.06 M phosphate buffer. Aliquots of 0.05 ml. were then applied to Whatman No. 1 filter paper and chromatographed by the descending method for 18 hours, using phenol saturated with water as solvent and water saturated with phenol to saturate the atmosphere of the tank. The papers were then removed and dried in air at room temperature (5). The position of the amino acids was located by spraying the paper with a 0.1 per cent solution of ninhydrin in butanol and heating gently with an infra-red lamp.

The areas of paper corresponding to glutamate were cut out, rolled, and placed in test tubes. Elution of the amino acid from the paper and quantitative color development with ninhydrin were performed in one operation by adding the reagents and treating the paper as described in the procedure of Troll and Cannan (6). Areas of paper containing standard amounts of glutamate were analyzed concomitantly. Papers corresponding to incubation mixtures containing 0.5 ml. of serum, or 1.0 ml. of hemolysate were used as blanks.

RESULTS

Reliability of the method

No loss of glutamate was encountered in the incubation period with serum or in the process of paper chromatography. Satisfactory recovery of known quantities of glutamate added to serum and incubated for 18 hours, or applied to paper directly, was obtained (Table I).

Presence and properties of the enzyme in serum

Incubation of aspartate and alpha-keto glutarate or alanine and alpha-keto glutarate, without addition of serum, failed to form detectable quantities of glutamate after 18 hours of incubation. Incubation of 0.5 ml. of serum and any one of the substrates singly, similarly showed no formation of measurable glutamate. Thus, non-enzymatic transamination was inferred not to occur under the conditions of these experiments, and concen-

TABLE I

Recovery of added glutamate

Aliquot	Micromoles added	Micromoles recovered	% Recovery
A. From paper after phenol chromatography			
1.	0.405	0.410	101.1
2.	0.405	0.405	100.0
3.	0.405	0.415	102.3
4.	0.405	0.424	104.7
B. From serum after eighteen-hour incubation			
1.	8.00	8.10	101.1
2.	8.00	8.57	107.0
3.	8.00	7.60	95.0

tration of alpha-keto glutarate and amino group donors in serum and hemolysates was inferred to be negligible. Incubation of 0.5 ml. of serum with aspartate and alpha-keto glutarate resulted in the formation of from 4.4 to 15.0 micromoles of glutamate in 18 hours, depending on the serum sample tested.

The amount of glutamate produced was found to be directly proportional to the time of incubation when identical mixtures of serum, buffer, and substrates were incubated for varied intervals of time (Figures 1 and 2). The rate of production of

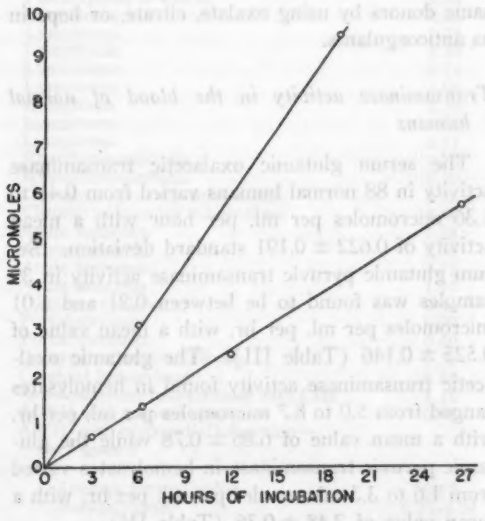

FIG. 1. TIME RATE OF GLUTAMATE PRODUCTION *Via* TRANSAMINATION (TWO 0.5 ML. SERUM SAMPLES)

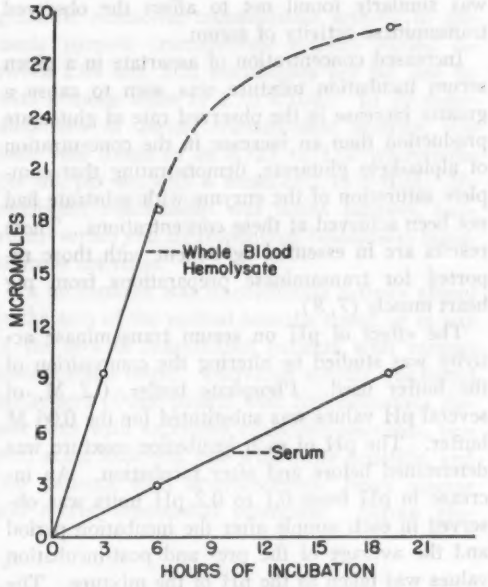

FIG. 2. RATE OF GLUTAMATE PRODUCTION *Via* TRANSAMINATION (WHOLE BLOOD HEMOLYSATE AND SERUM COMPARED)

glutamate was seen to be directly proportional to the quantity of serum, when 0.5 ml. and 1.0 ml. samples of the same sera were incubated with identical substrate mixtures (Table II).

Pyridoxal phosphate has been shown to act as coenzyme in transamination reactions and O'Kane and Gunsalus have determined the coenzyme saturated curve (7). Addition of a buffered solution of pyridoxal phosphate in a concentration of ten micrograms per ml. was found to have no measurable effect on the transaminase activity of the serum. Addition of a boiled and filtered extract of rat liver, used as source of possible activators,

TABLE II

The effect of enzyme concentration on rate of transamination incubation period—twenty hours

Sample	Micromoles glutamate produced by 0.5 ml.	Rate *micromoles/ml./hr.*	Micromoles glutamate produced by 1.0 ml.	Rate *micromoles/ml./hr.*
1.	5.6	0.56	10.4	0.52
2.	4.9	0.49	9.7	0.48
3.	6.6	0.66	11.0	0.55
4.	6.4	0.64	11.7	0.59
5.	5.9	0.59	12.3	0.61
6.	5.2	0.52	9.8	0.49

was similarly found not to affect the observed transaminase activity of serum.

Increased concentration of aspartate in a given serum incubation mixture was seen to cause a greater increase in the observed rate of glutamate production than an increase in the concentration of alpha-keto glutarate, demonstrating that complete saturation of the enzyme with substrate had not been achieved at these concentrations. These results are in essential agreement with those reported for transaminase preparations from pig heart muscle (7, 8).

The effect of pH on serum transaminase activity was studied by altering the composition of the buffer used. Phosphate buffer, 0.2 M, of several pH values was substituted for the 0.06 M buffer. The pH of each incubation mixture was determined before and after incubation. An increase in pH from 0.1 to 0.2 pH units was observed in each sample after the incubation period and the average of the pre- and post-incubation values was taken as the pH of the mixture. The finding of maximal activity between pH 7.0 and 8.0 (Figure 3) is in essential agreement with the results of Cohen (9) and others using pig heart muscle as source of transaminase.

No change in transaminase activity with time was noted in serum samples stored from ten minutes to 96 hours at room temperature, or for periods of from one hour to two weeks in the refrigerator (0 to 5°C.). The transaminase activity was not changed by freezing or lyophilization of the serum. No change in activity was noted in sera subjected to 56°C. for 25 minutes. Sera heated to 100°C. for ten minutes were found to have a decrease in activity to 10 per cent of the original transaminase activity.

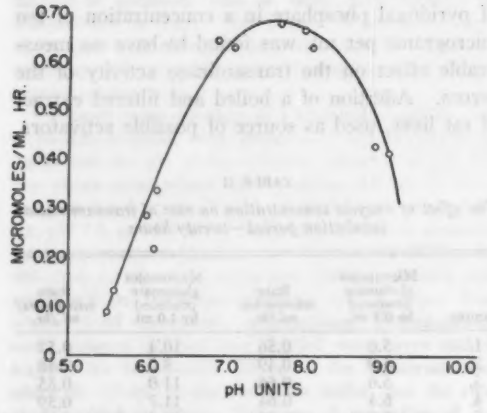

FIG. 3. EFFECT OF VARYING HYDROGEN ION CONCENTRATIONS ON SERUM TRANSAMINASE ACTIVITY

TABLE III

Distribution of transaminase activity in serum of healthy adults

Transaminase activity *micromoles/ml./hr.*	Aspartate-alpha-keto glutarate No. of samples	Aspartate-alpha-keto glutarate %	Alanine-alpha-keto glutarate No. of samples	Alanine-alpha-keto glutarate %
0.20–0.39	0	0	2	5.1
0.40–0.49	7	7.8	11	28.2
0.50–0.59	22	25.0	16	41.1
0.60–0.69	24	27.2	5	12.5
0.70–0.79	15	17.1	2	5.1
0.80–0.89	5	5.7	1	2.5
0.90–0.99	9	10.2	1	2.5
1.00–1.09	5	5.7	1	2.5
1.36	1	1.1		
Total number tested	88	100	39	100
Mean transaminase activity *micromoles/ml./hr.*	Aspartate-alpha-keto glutarate	0.622±0.191 Std. deviation		
	Alanine-alpha-keto glutarate	0.525±0.146 Std. deviation		

No difference could be detected between transaminase activity in serum and in plasma from the same donors by using oxalate, citrate, or heparin as anticoagulants.

Transaminase activity in the blood of normal humans

The serum glutamic oxalacetic transaminase activity in 88 normal humans varied from 0.41 to 1.36 micromoles per ml. per hour with a mean activity of 0.622 ± 0.191 standard deviation. Serum glutamic pyruvic transaminase activity in 39 samples was found to be between 0.21 and 1.01 micromoles per ml. per hr. with a mean value of 0.525 ± 0.146 (Table III). The glutamic oxalacetic transaminase activity found in hemolysates ranged from 5.0 to 8.7 micromoles per ml. per hr. with a mean value of 6.86 ± 0.78 while the glutamic pyruvic transaminase in hemolysates varied from 1.6 to 3.3 micromoles per ml. per hr. with a mean value of 2.48 ± 0.36 (Table IV).

In each of 29 hemolysate samples, the ratio of

TABLE IV

Distribution of transaminase activity in whole blood hemolysates of healthy adults

Aspartate-alpha-keto glutarate			Alanine-alpha-keto glutarate		
Transaminase activity *micromoles/ml./hr.*	No. of samples	%	Transaminase activity *micromoles/ml./hr.*	No. of samples	%
5.0–5.9	3	10.3	1.5–1.9	3	10.3
6.0–6.9	12	41.4	2.0–2.5	12	41.4
7.0–7.9	12	41.4	2.5–3.0	11	38.0
8.0–8.9	2	6.9	3.0–3.3	3	10.3
Total No.	29	100.0	Total No.	29	100.0
Mean transaminase activity *micromoles/ml./hr.*		Aspartate-alpha-keto glutarate	6.86±0.78 Std. deviation		
		Alanine-alpha-keto glutarate	2.48±0.36 Std. deviation		

glutamic oxalacetic transaminase activity to glutamic pyruvic transaminase activity varied between 2.04 and 3.60 micromoles per ml. per hr. with a mean value of 2.70 ± 0.40. In 39 determinations of serum transaminase activity, this ratio was found to be 0.725 to 1.67 micromoles per ml. per hr. with a mean of 1.15 ± 0.23. No consistent relationship was noted between individual serum and corresponding hemolysate transaminase activities.

In no instance was transaminase activity absent in the sera of the normal humans tested or in any of the sera of hospitalized patients with various disease states tested. Increased activity was found in the sera of one patient with lymphomatous disease, one with extensive rhabdomyosarcoma, two with acute leukemia, one with acute hepatitis, and two patients with arteriosclerotic heart disease and recent myocardial infarction. Serum glu-

TABLE V

Transaminase activity in blood of hospitalized patients in micromoles per ml. per hr.

Clinical diagnosis	Serum transaminase glutamic		Hemolysate transaminase glutamic	
	Oxalacetic	Pyruvic	Oxalacetic	Pyruvic
Carcinoma of lung	0.54	0.41	8.86	2.07
Carcinoma of lung	0.51	0.38	1.73	2.54
Carcinoma of palate	0.89	0.70	6.25	2.60
Carcinoma of eyelid	0.75	0.47	8.05	1.80
Carcinoma of breast	0.80	0.73		
Fibromyoma of uterus	0.81	0.59	8.75	2.73
Lymphoma	0.46	0.51	11.3	2.70
Lymphoma	0.71	0.47	7.85	1.56
Lymphoma	0.52	0.39	6.45	2.53
Lymphoma	0.74	0.53	6.80	1.66
Lymphoma	1.06	0.70	6.35	2.00
Lymphoma	0.36	0.32	7.00	1.60
Lymphoma	0.47	0.37		
Lymphoma	0.71	0.48		
Rhabdomyosarcoma	1.70	1.60	9.13	7.46
Rhabdomyosarcoma	0.40	0.35	7.26	2.00
Chronic pulmonary tuberculosis	0.92	1.24	7.20	3.60
Chronic pulmonary tuberculosis	0.58	0.44	8.95	1.60
Acute leukemia	1.28	1.51	5.26	3.40
Acute leukemia	0.36	0.55	5.20	1.60
Acute leukemia	1.72	1.25	7.95	3.73
Acute hepatitis	1.97	1.75	7.20	10.4
Diabetes mellitus	0.46	0.35	8.46	2.73
Portal cirrhosis	0.99	1.28	8.70	3.50
Portal cirrhosis	0.49	0.38	3.10	1.40
Generalized arteriosclerosis	0.50	0.63	6.13	3.26
Cerebral hemorrhage	0.85	0.41	9.40	3.40
Arteriosclerotic heart disease with CHF	1.16	0.81	5.00	2.20
ASHD with anginal syndrome	0.95	1.25	8.65	3.27
ASHD with myocardial infarction—see Figure 5				
Average normal value	0.62	0.52	6.86	2.48
Normal range*	0.24–1.04	0.23–0.82	5.30–8.42	1.76–3.20

* Normal range represents the normal mean value plus or minus two standard deviations.

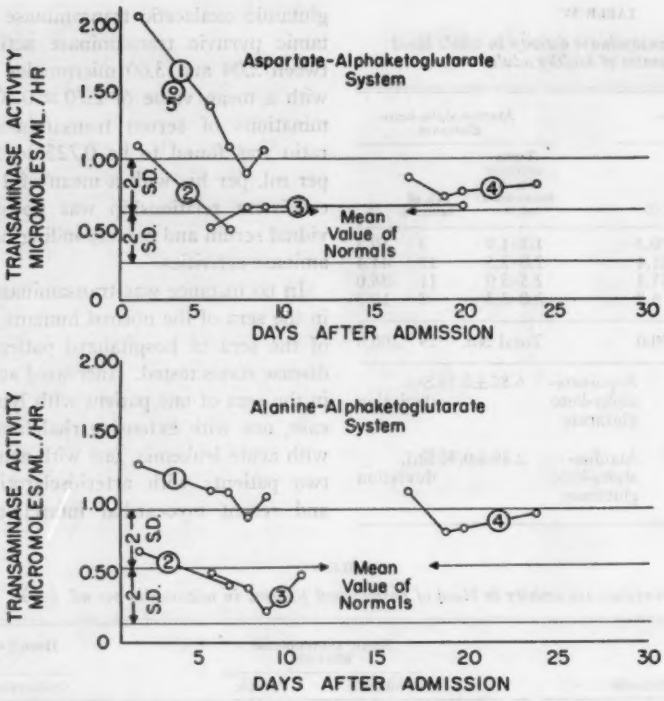

FIG. 4. SERUM TRANSAMINASE ACTIVITY IN PATIENTS WITH ADMISSION DIAGNOSIS OF MYOCARDIAL INFARCTION

tamic oxalacetic transaminase activity and serum glutamic pyruvic transaminase activity was seen to vary together in most cases of marked departure from the normal range. Somewhat greater variation was found in the transaminase activity of the hemolysates from the same patients (Table V).

Figure 4 represents serial determinations of serum transaminase activity in five patients admitted to the hospital with the admitting diagnosis of acute myocardial infarction at various times after the onset of acute infarction. Of the five patients shown, the clinical picture subsequent to admission substantiated the diagnosis of transmural myocardial infarction in Nos. 1, 2, 4, and 5. Elevated values were found in patients Nos. 1 and 5, the only patients with acute infarctions studied during the first week after infarction. The finding of 2.02 units in patient No. 1 represents the highest value found in any of the samples tested and almost twice the value taken as the upper limit of normal.

DISCUSSION

The method of transaminase assay by quantitative paper chromatography of the glutamate produced was chosen because of its sensitivity and the simplicity of the equipment required. Other methods of measuring transamination reactions may be divided into two main types: Those utilizing either specific chemical or enzymatic decarboxylation of one of the products and subsequent manometric measurement of carbon dioxide evolved and those utilizing the high ultraviolet absorption of oxalacetate at wavelength 280 millimicra to follow the glutamic oxalacetic transaminase reaction by measuring a change in optical density as oxalacetate is produced or consumed. The high bicarbonate content of blood together with low transaminase activity makes a method depending on carbon dioxide evolution difficult to apply to serum. Low transaminase activity and high protein content together with the known instability of oxalacetate make the usual spectrophotometric assay difficult to apply to serum.

The results demonstrated the enzymatic nature of the observed transamination and suggest that serum glutamic oxalacetic transaminase has similar chemical characteristics to transaminase derived from other sources. The failure of pyridoxal phosphate or boiled liver extract to increase the measured transaminase activity may be taken as evidence that the enzyme was fully activated in the serum samples tested. The difference in comparative amounts of the two transaminases in serum and hemolysates could represent a difference in the rates of diffusion of the two enzymes across the cellular membranes of the blood cells, or, as seems more likely, this difference could be taken as suggestive evidence for a source of the serum enzymes apart from the blood cells.

The sera of patients with acute myocardial infarction were thought worthy of particular attention because of the possibility that destruction of cardiac muscle, reported rich in transaminase activity, might result in a release of this enzyme into the blood stream and might thus increase the serum transaminase activity. The finding of increased serum transaminase activity in the two patients studied during the first week after infarction is compatible with such a possibility.

SUMMARY

1. The presence of glutamic oxalacetic and glutamic pyruvic transaminase activity in human serum, plasma, and whole blood hemolysates has been demonstrated. A method is presented for their measurement by estimating the rate of glutamate formation employing quantitative paper chromatography.

2. The chemical properties of the enzyme in serum, including the variation in activity with changes in concentration of enzyme, substrate, and hydrogen ion were found similar to those reported for transaminases in animal tissues. Freezing or lyophilizing the serum, or storing it at 0 to 5°C. for periods up to two weeks resulted in no significant change in transaminase activity. Boiling destroyed the activity.

3. Glutamic oxalacetic and glutamic pyruvic transaminase activities were found to be approximately equal in normal human serum. Whole blood hemolysates were found to have ten times as much glutamic oxalacetic transaminase activity as serum. Glutamic oxalacetic transaminase was found to be approximately 2.7 times as active as glutamic pyruvic transaminase in hemolysates.

4. A survey of transaminase activity in the blood of hospitalized patients revealed departure from the normal range of activity in several disease states.

ACKNOWLEDGMENT

We wish to acknowledge gratefully the interest, advice and assistance of Drs. Aaron Bendich, Oscar Bodansky, Severo Ochoa, and Rulon Rawson.

APPENDIX

A NOTE ON THE SPECTROPHOTOMETRIC ASSAY OF GLUTAMIC-OXALACETIC TRANSAMINASE IN HUMAN BLOOD SERUM

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A spectrophotometric method was devised in which the transamination reaction (Reaction 1) is coupled to the reduction of oxalacetate to malate by reduced diphosphopyridine nucleotide (DPNH), in the presence of an excess of purified malic dehydrogenase (Reaction 2). The oxidation of DPNH, and therefore the transamination reaction, is followed by measuring the decrease in light absorption at wave length 340 mμ, at which the reduced pyridine nucleotides have an absorption peak.

(*1*) α-Keto glutarate + aspartate ⇆ L-glutamate + oxalacetate

(*2*) Oxalacetate + DPNH + H^+ ⇄ L-malate + DPN^+

METHODS

Materials. Aspartic acid, alpha-keto glutaric acid and reduced diphosphopyridinenucleotide were obtained commercially. Purified malic dehydrogenase, prepared from pig heart muscle by the method of Straub (10), was tested and found free of detectable transaminase or glutamic dehydrogenase activity.[1]

Experimental procedure. From 0.1 to 1.0 ml. of serum, 1.0 ml. of 0.1 M phosphate buffer, pH 7.4, 0.5 ml. of 0.2 M aspartate in buffer, pH 7.4, 0.2 ml. of DPNH (1 mgm. per ml.) and 0.1 ml. of a solution of purified malic dehydrogenase (50 micrograms of enzyme protein per ml.) were mixed and brought to a final volume of 2.8 ml. in a cuvette having a 1.0 cm. light path. The blank contained all reactants listed except DPNH. After 10 minutes, 0.2 ml. of 0.1 M alpha-keto glutarate in buffer, pH 7.4, was added. The optical density at wavelength 340 mμ was followed for five minutes, and the rate of decrease of optical density taken as the measure of the transaminase activity of the serum. The reaction was followed in a Beckman model DU spectrophotometer at room temperature. The activity is expressed as units per ml. of serum per minute. One unit equals a decrease in optical density of 0.001 under the conditions described.

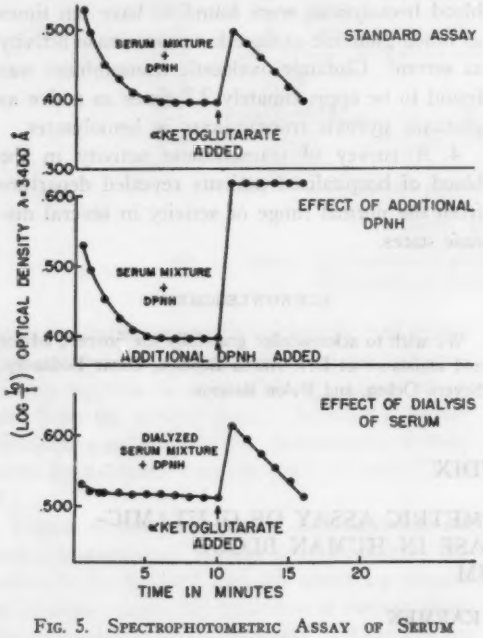

FIG. 5. SPECTROPHOTOMETRIC ASSAY OF SERUM TRANSAMINASE

Top and bottom curves, all components except α-keto glutarate present initially; α-keto glutarate added at time indicated by arrows. Middle curve: all components present initially, additional DPNH at time indicated by arrow. DPNH added at zero time in all cases. The increase in optical density when α-keto glutarate is added is due to the absorption of light by α-keto glutarate at this wavelength.

[1] I am indebted to Dr. Martin Schwartz for this preparation.

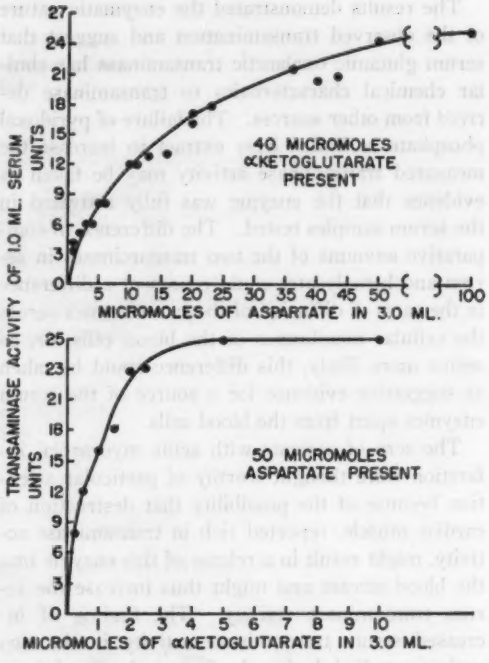
TRANSAMINASE ACTIVITY OF 1.0 ML. SERUM UNITS
40 MICROMOLES αKETOGLUTARATE PRESENT
MICROMOLES OF ASPARTATE IN 3.0 ML.
50 MICROMOLES ASPARTATE PRESENT
MICROMOLES OF αKETOGLUTARATE IN 3.0 ML.

FIG. 6. SERUM TRANSAMINASE ACTIVITY AS A FUNCTION OF SUBSTRATE CONCENTRATION

Comments on procedure. When DPNH is added to serum without addition of substrates the optical density of the mixture decreases for six to seven minutes, indicating the oxidation of a finite quantity of DPNH. After this reaction has stopped, completion of the transamination system results in a steady decrease in optical density which is taken as a measure of the transaminase activity of the serum (Figure 5, top curve).

The rate or amount of DPNH oxidation by serum alone is not changed by the addition of malic dehydrogenase and either aspartate or alpha-keto glutarate. The amount oxidized is not appreciably affected by adding more DPNH (Figure 5, middle curve). Addition of 0.2 ml. of a solution of potassium pyruvate (4 mgm. per ml.), after this reaction has stopped, results in complete oxidation of the DPNH present at a measurable rate, demonstrating the presence of lactic dehydrogenase in serum. Dialysis of the serum against 0.1 M phosphate buffer reduces the amount of DPNH oxidized without changing the measurable transaminase activity of the serum (cf. Figure 5, top and bottom curves).

It is, therefore, suggested that the oxidation of DPNH by serum alone depends on the presence of pyruvate and lactic dehydrogenase in serum. The reaction stops when all the pyruvate is enzymatically reduced to lactate by DPNH.

Omitting malic dehydrogenase from otherwise complete transamination reaction mixtures resulted in a variable decrease in the observed rate of DPNH oxidation. That the reaction was still measurable was taken as suggestive evidence either for the presence of malic dehydrogenase in serum or for the decarboxylation of oxalacetate to pyruvate and subsequent DPNH oxidation by lactic dehydrogenase. Addition of more malic dehydrogenase to any serum transaminase assay resulted in no further increase in the measured rate of reaction, indicating that the enzyme was present in excess in the assay mixture.

RESULTS

Study of the variation of serum transaminase activity with substrate concentration indicated that maximal activity was obtained when the three ml. reaction mixture contained more than 50 micromoles of aspartate and 5 micromoles of alpha-keto glutarate (Figure 6). One hundred micromoles of aspartate and 20 micromoles of alpha-keto glutarate were used in subsequent determinations.

Proportionality of the observed rate of reaction to the amount of serum present was observed over a wide range of serum transaminase activities (Figure 7).

Transaminase activity was measured in the sera of 50 normal humans in addition to those determined by the chromatographic assay method. The values found ranged from a low of nine to a high of thirty-two units per ml. per minute with a mean value of 19.6. Conversion of these units to micromoles per ml. per hour, using the extinction coefficient for DPNH determined by Horecker and Kornberg (11) gives a mean value of 0.57, which falls within the range of 0.24 to 1.04 micromoles per ml. per hour found by the quantitative paper chromatographic assay.

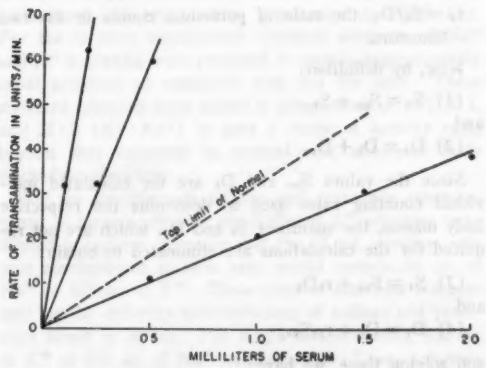

FIG. 7. TRANSAMINASE ACTIVITY AS A FUNCTION OF SERUM CONCENTRATION

Three different serum samples.

ACKNOWLEDGMENT

The author wishes to express his appreciation to Dr. Severo Ochoa for invaluable help in performing this work.

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AN IMPROVED METHOD FOR SIMULTANEOUS DETERMINATION OF EXCHANGEABLE BODY SODIUM AND POTASSIUM

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In a previous paper (1) a method was reported for the simultaneous determination of exchangeable body sodium and potassium by the isotope dilution technique in which the two isotopes are separated by ion exchange chromatography.[1] The method presented here uses a differential counting technique [2] to measure the amounts of each isotope without separation, thus effecting a considerable simplification.

Potassium 42 has considerably harder β-rays than does sodium 24, whereas sodium 24 emits many more γ-rays.[3] In consequence a suitable pair of detectors for the differential counting of these isotopes is a G. M. dip counter for β-ray detection and a well-type scintillation counter for γ-ray detection. With the particular technique employed, the counting rates for serum samples labelled with Na^{24} are approximately 1.8 times greater in the scintillation than in the dip counter, whereas serum labelled with K^{42} counts approximately 10 times more in the dip than in the scintillation counter. The accurately determined values of these two ratios can be used to calculate the separate activities in any mixed sample from its observed counting rates in the two detectors making it possible to determine exchangeable body sodium and potassium simultaneously without any separation of the ions from one another.

The accuracy and practicability of the method were tested *in vitro* by comparison of the assays of mixtures by differential counting with the known values; and *in vivo* by comparison of the values of exchangeable body sodium and potassium obtained simultaneously by this method on seven normal subjects with separate values obtained on the same subjects. The results compare favorably with those obtained by more complex techniques.

THE EQUATIONS FOR DIFFERENTIAL COUNTING

To express the relationships which exist among the various counting rates, the following symbols are used:

S_{Na} = the scintillation counting rate of the Na^{24},
S_K = the scintillation counting rate of the K^{42},
S_t = the total scintillation counting rate of the mixture,
D_{Na}, D_K and D_t are the corresponding quantities for the dip counter,
$r_{Na} = D_{Na}/S_{Na}$, the ratio of sodium counts in the two detectors, and
$r_K = S_K/D_K$, the ratio of potassium counts in the two detectors.

Now, by definition:

(1) $S_t = S_{Na} + S_K$

and

(2) $D_t = D_K + D_{Na}$.

Since the values S_{Na} and D_K are the calculated individual counting rates used to determine the respective body masses, the quantities S_K and D_{Na} which are not required for the calculations are eliminated to obtain:

(3) $S_t = S_{Na} + r_K D_K$

and

(4) $D_t = D_K + r_{Na} S_{Na}$,

and solving these, we have:

(5) $S_{Na} = [1/(1 - r_{Na}r_K)]S_t - [r_K/(1 - r_{Na}r_K)]D_t$

and

(6) $D_K = [1/(1 - r_{Na}r_K)]D_t - [r_{Na}/(1 - r_{Na}r_K)]S_t$.

After the ratios have been accurately measured, their values are substituted in the brackets to give numerical

[1] A discussion of the general principles underlying the application of the isotope dilution technique to the measurement of body constituents may be found in the paper by Edelman and his co-workers (2). The physiologic implications and clinical applications of body electrolyte measurements are discussed by Corsa and his co-workers (3), Arons, Vanderlinde, and Solomon (1) and Forbes and Perley (4).

[2] Two other types of differential counting of K^{42} and Na^{24} are reported by Tait and Williams (5) and by Burrows, Hine, and Ross (6).

[3] (7)

	Half life	β-rays	γ-rays	%
Na^{24}	15.04 hours	1.4 Mev	2.8 and 1.4 Mev	100
K^{42}	12.44 hours	3.6 Mev	none	80
		2.1 Mev	1.5 Mev	20

coefficients. In most of the present work, for example, the values are $r_{Na} = 0.541$ and $r_K = 0.0986$, giving rise to the final equations:

(7) $S_{Na} = 1.056\ S_t - 0.1043\ D_t$

and

(8) $D_K = 1.056\ D_t - 0.571\ S_t$.

The values S_{Na} and D_K can be calculated readily for any mixed sample by substitution of the two observed counting rates, S_t and D_t. These equations apply only to the pair of detectors given in this example. Any change of either detector, such as replacement of the dip tube, requires the determination of new values of the ratios to give new equations similar to *7* and *8*.

The equations are strictly correct only if the counts are made simultaneously with both detectors since the half-lives of the two isotopes are different [4] so that it is impossible to apply exact decay corrections to the observed counting rates of the mixture. If the counting is arranged, however, so that no more than ten minutes elapses between the mid-times of determination of S_t and D_t, no significant error is introduced by correcting the scintillation count with a Na^{24} decay factor to the dip counting time to give the values of S_{Na} and D_K at that time.

The derived scintillation rate S_{Na} is used together with the injected sodium counts as measured with the scintillation counter in the calculation of the exchangeable body sodium and similarly the derived dip counting rate D_K is used together with the injected potassium counts as measured in the dip counter to calculate the exchangeable body potassium.

EXPERIMENTAL METHOD

Sodium 24 and potassium 42 were obtained from the Atomic Energy Commission at the Brookhaven National Laboratory and prepared as previously described (1). For the *in vitro* experiments standard solutions of Na^{24} and K^{42} in plasma were prepared to contain approximately equal activities as measured with the dip tube. These standard solutions were mixed in proportions of 1:1, 3:1, and 25:3 (K^{42}:Na^{24}) to give a range of activity ratio beyond that expected in normal and abnormal body electrolyte conditions.

For the simultaneous sodium and potassium measurements in human subjects, the Na^{24} and K^{42} solutions were diluted in isotonic saline so that the 50 ml. injection volume administered in each case would contain 10 μc. of Na^{24} or 350 μc. of K^{42}. These relative activities compensate for the different concentrations of sodium and potassium found in serum. For single measurements, 350 μc. of K^{42} or 125 μc. of Na^{24} were injected. These solutions were neutralized, sterilized, and injected serially *via* the tubing of a 5 per cent dextrose-in-water intravenous infusion. All radioactivity injections were of 50 ml. volume from calibrated syringes to minimize the error of injection which was found to be about 0.3 per cent. After a 24-hour equilibration period during which all urine was collected, 50 ml. of blood were drawn and the serum separated by centrifugation. The amount of radioactivity lost in feces and sweat is insignificant in normal subjects (1).

To measure the injected radioactivity, duplicate standard solutions of Na^{24} (dilution 10:250) were counted in triplicate for 10,000 counts in the scintillation counter and K^{42} (dilution 1:1000) standards were similarly assayed in the dip counter. Serum samples for the *in vivo* measurements and the *in vitro* mixtures were counted in duplicate to 10,000 counts in both scintillation and dip counters. For single determinations of the exchangeable body sodium, measurements were made in the scintillation counter; similarly for single potassium determinations the dip counter was used. Urine was counted in duplicate to 5000 counts in each detector. The usual corrections for decay, background and dead time were made.

The whole body radiation resulting from the injections of radioactivity in the combined experiment, as computed by the method of Marinelli, Quimby, and Hine (8), is 0.45 equivalent roentgens; the dose is appreciably less when only one isotope is used. Since this combined dose was used only once and the single dose twice in these experiments, the total body radiation administered was considered to be well within tolerance limits.

The sodium and potassium concentrations in each serum sample were determined in duplicate on each of two days on a Perkin-Elmer flame photometer utilizing an internal standard. The average values were used in the calculations of the exchangeable body sodium and potassium. These data, together with the corrected activity values were substituted in the isotope dilution formula [5] to give the exchangeable body sodium and potassium.

COUNTING EQUIPMENT

The dip counter

The type of tube used in this work [6] is the same as was employed previously when the isotopes were separated by ion-exchange chromatography. A section of the dip counter, cup and supports is shown in Figure 1. The upper, fixed support holds the tube rigidly and receives the top of the brass cup. A hinged, lower support terminates in a conical point which engages the bottom of the cup. The two supports serve to position the cup in a positive

[4] See Footnote 3.

[5] Total exchangeable electrolyte = $\frac{\text{(activity injected} - \text{activity excreted)}}{\text{(activity in body fluid sample/electrolyte in sample)}}$.

[6] Described in reference (9) and manufactured by Radiation Counter Laboratories as Mark I, Model 80A. The tube is 105 mm. long by 18 mm. in diameter with a sensitive region 36 mm. long and a glass wall 30 mg./cm^3. in thickness. This tube is somewhat photosensitive and the portions exposed to light should be coated with black paint.

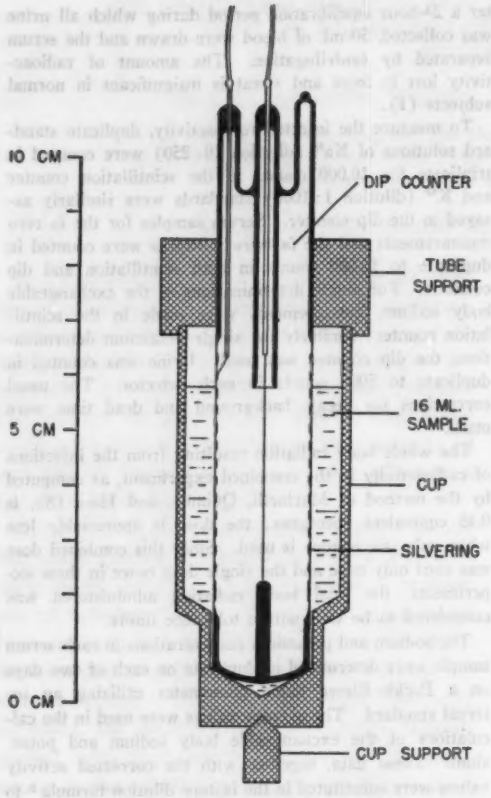

FIG. 1. A SECTION OF THE DIP COUNTER, CUP AND SUPPORTS

and reproducible manner. This construction, together with the practice of using the same cup for all measurements, insures a constant geometry of solution. A larger diameter was chosen for the present cup in order to favor the detection of K^{42} over Na^{24} even though a larger serum sample, 16 ml., is required. This amount need not be pipetted to better than ± 0.1 ml. since it exceeds the value, 15.5 ml., above which all volumes of any sample are found to give nearly the same counting rate.

In order to check the reproducibility of dip counting, measurements were made on a set of twelve identical serum samples labelled with K^{42}. The total count on each sample was about 12,000 and the coefficient of variation was found to be 1.03 per cent which compares favorably with the 0.91 per cent expected from the counting statistics.

The normal background of the counter with the cup filled and in position but without further shielding is 10 to 16 c/m. During a series of measurements the cup and counter are washed and wiped between each sample but, nevertheless, a gradual increase in background to as much as 100 per cent due to contamination may be observed. The background value, however, may be determined at intervals to sufficient accuracy so that the error of its determination does not contribute significantly to the calculated net counts of any of the samples or standards which are measured.

The scintillation counter

This apparatus consists of a commercial, well-type, scintillation unit [7] surrounded by 3 inches of lead shielding. The crystal is of a standard type,[8] 1 and ¾ inches in diameter, 2 inches high and containing a well ¾ inch in diameter and 1 and ½ inches deep. The well accommodates a sample of 2 ml. volume contained in a standard 13 by 100 mm. test tube [9] and is lined by a 1/16 inch thickness of steel to insure absorption of the β-particles.

The scintillation counter is especially convenient compared to the dip counter, since no decontamination is required between samples. The background has a relatively steady value but is high enough (104 c/m in our present apparatus) so that the statistical error in its determination contributes significantly to the error of the net count. Account is taken of this in the calculations of the errors shown in Tables I and II.

The 2 ml. volume needs to be pipetted accurately as the counting rate is directly proportional to the activity in the test tube. Ten identical samples of serum labelled with Na^{24}, each counted to 11,500 counts, gave a coefficient of variation of 1.12 per cent with 0.93 per cent expected from the counting statistics.

TABLE I

A comparison of known and simultaneously determined activities of in vitro mixtures

Mixture ratio K^{42}/Na^{24}	Counter and Isotope*	Activity known from separate counting† *counts per minute*	Activity determined by differential counting *counts per minute*	Diff.
1 to 1	S_{na}	2712±13‡	2720±18	+08
	D_k	1750±10	1727±28	−23
3 to 1	S_{na}	1356± 7	1341±11	−15
	D_k	2625±15	2657±25	+32
25 to 3	S_{na}	582± 3	584± 9	+02
	D_k	3126±18	3138±25	+12

* S_{na} denotes the scintillation sodium count and D_k denotes the G. M. dip potassium count.

† These counts are calculated from the mixture ratios and from the measured activities of the original K^{42} and Na^{24}-labelled plasma standards for which $D_k = 3501 \pm 20$ c/m and $S_{na} = 5425 \pm 26$ c/m, respectively.

‡ The errors shown are the standard deviations expected from the counting statistics in counts per minute.

[7] N. Wood Laboratories, SC-2L.

[8] Thallium activated sodium iodide manufactured by The Harshaw Chemical Co.

[9] Rimless culture tube Corning No. 9820.

TABLE II

Two examples showing observed and derived counting rates together with their standard deviations due to counting statistics for cases (a) normal and (b) abnormal

	A. Normal*				B. Abnormal*			
	Dip	S.D. %	Scin.	S.D. %	Dip	S.D. %	Scin.	S.D. %
Total counts (Approx.)	20,000	0.7	22,000	0.7	21,000	0.7	20,000	0.7
Time (Approx.)	50 m	—	56 m	—	30 m	—	50 m	—
Total observed rate, c/m†	393	0.7	406	0.7	724	0.7	402	0.7
Background, c/m	15	4.7	149	1.0	17	2.4	110‡	0.9
Net observed rates D_t and S_t, c/m	378	0.8	257	1.2	707	0.7	292	1.0
Derived rates D_k and S_{Na}, c/m	253	1.4	232	1.5	580	0.9	235	1.4

* Case A is J. V. of Table III with values Na_e = 3,179 mEq. and K_e = 4,035 mEq. with Na_e/K_e ratio 0.79. The range of ratios for the seven normal subjects of Table III is 0.74 to 0.94. Case B has values Na_e = 3,955 mEq. and K_e = 2,305 mEq. with an abnormal ratio of 1.72.

† Counts per minute.

‡ The improvement in this background is due to additional shielding.

SOURCES OF ERROR

Errors due to counting statistics

In evaluating any differential counting technique, one important consideration is the cumulative effect of the errors of measurement on the derived quantities; in this instance, the effect on S_{Na} and D_K of the errors in determining r_{Na}, r_k, S_t, and D_t.

The errors introduced by the uncertainty of the values of r_{Na} and r_k should be rendered insignificant by making sufficiently long counts on standard solutions so that their values are known to better than ½ per cent. In our work, this is done whenever a new detector is put into service. In addition the ratios are given a rough check each time body mass measurements are made, by counting each of the injection standards in both counters. If each detector is provided with a long-lived standard, such as a tube of uranium salt with reproducible geometry, a further check on the constancy of the detectors may be made by counting these standards with each set of determinations.

TABLE III

A comparison of simultaneous measurements of exchangeable body sodium and potassium with single determinations in normal male subjects

Subject	Age	Weight	Exchangeable* body Na	Difference from mean as % of mean	Exchangeable* body K	Difference from mean as % of mean
	(*yrs.*)	(*Kg.*)	(*mEq.*)		(*mEq.*)	
B. B.	28	92.0	3,243 3,129	+1.8	4,084 3,910	+2.2
K. O'D.	19	89.1	3,648 3,421	+3.2	4,266 4,440	−2.0
A. R.	30	83.8	3,158 3,157	0.0	4,053 3,714	+4.4
J. H.	26	90.0	3,379 3,132	+3.8	4,243 4,260	−0.2
E. P.	26	62.3	2,730 2,648	+1.5	2,984 2,814	+2.9
J. V.	30	85.0	3,088 3,179	−1.5	3,862 4,035	−2.2
J. W.	29	80.0	3,036 3,133	−1.6	3,855 3,684	+2.3
		Coefficient of variation		3.2%		3.6%

* Results of the single measurements are listed first and the simultaneously determined values second. The two single and the simultaneous measurements were carried out in most instances at seven-day intervals on three successive weeks.

Application of the rules for the accumulation of errors to equations *5* and *6* gives expressions for the errors in D_k and S_{Na}. The terms containing r_k^2 and those containing the squares of the errors of r_k and r_{Na} may be neglected, if one assumes the degree of accuracy discussed above. The simplified expressions are:

(*9*) $d(S_{Na}) = [1/(1 - r_k r_{Na})] \times d(S_t)$

and

(*10*) $d(D_k) = [1/(1 - r_k r_{Na})] \times [r_{Na}^2 \times d^2(S_t) + d^2(D_t)]^{1/2}$,

where the symbol d(X) designates the standard deviation of X in counts per minute. For the values $r_k = 0.10$ and $r_{Na} = 0.54$, the equations become:

(*11*) $d(S_{Na}) = 1.06\ d(S_t)$

and

(*12*) $d(D_k) = 1.06 \times [0.29\ d^2(S_t) + d^2(D_t)]^{1/2}$.

In order to illustrate the accumulation of errors in solving the equations, two cases have been chosen, one a normal subject and the other a patient exhibiting an unusually high body Na/K ratio (see Table II). Approximately the same doses of 350 μc. of K^{42} and 10 μc. of Na^{24} were given to each subject. The counts have been corrected to a single time in each case and the derived rates and their errors calculated from equations *11* and *12*.

The results show that the errors in the derived quantities, S_{Na} and D_k, are approximately 1.4 per cent which is twice the 0.7 per cent of the observed counts. This increase depends not only on the accumulation of errors in solving the equations but also on a significant contribution from the uncertainty in the value of the background of the scintillation counter. The background of the dip counter is low enough not to contribute significantly. The counting errors are found to be no greater for the abnormal than for the normal subject.

The urine samples are generally counted in duplicate to 5,000 counts in each detector. Because of the preponderance of potassium activity, the sodium determination may have a rather large percentage error. Since the excreted activity represents only about 5 per cent of the injected activity for each isotope, the inaccuracies thus introduced into the final body electrolyte values are negligible.

The injection standards are counted to ½ per cent accuracy. When this error is included about 0.1 per cent is added to each of the errors shown for the derived quantities. Thus the maximum overall counting error found in these examples is 1.6 per cent, with 1.4 per cent as an average value.

The effect of specific gravity

The specific gravity of the sample affects the counting rate of the dip counter, but not of the scintillation counter. Account must be taken of this effect in determining the exchangeable body potassium, since an aqueous, rather than a serum, dilution of the injected material is used. The dip count obtained from this standard must be decreased by a small amount to give the serum-equivalent figure for use with the D_k value in the calculation of exchangeable body potassium. In the corresponding sodium calculation the scintillation count on the aqueous standard is used as serum-equivalent without correction.

The magnitude of the specific gravity correction factor, as determined from measurements by us on serum and sucrose solutions, is $1 + 0.7\ (\eta - 1)$ for K^{42} in a medium of specific gravity η. For example, with the average serum value $\eta = 1.025$ the K^{42} dip counting rate is 1.8 per cent lower than it would be in aqueous solution. The effect on Na^{24} is approximately the same.

Although the ratios r_{Na} and r_k are affected by variations in specific gravity, the values determined for serum of average specific gravity are satisfactory for the activity calculations in all but exceptional cases. A deviation of specific gravity downward 1 per cent from normal will cause the equations to give values of S_{Na} which are approximately 0.1 per cent too low and values of D_k which are approximately 0.3 per cent too high. Equal and opposite errors in these quantities are caused by a specific gravity 1 per cent higher than normal. Since a 1 per cent deviation is unusual and since the errors introduced are small compared to other errors in the measurements, it is not likely that a case will be encountered for which it would be worthwhile to recalculate the values of r_{Na} and r_k and set up new equations.

Summary of expected errors in determining exchangeable body sodium and potassium

From an analysis of the pairs of flame photometer values measured for each electrolyte, the average values, used in the final calculations, are estimated to have standard deviations of 0.5 per cent for sodium and 1.0 per cent for potassium.

The contributions of the following effects are estimated to be negligible for normal subjects: errors in urine values, errors in dilutions of the standards, and errors due to variations of the specific gravity of the serum.

In summary, the expected errors for normal subjects for sodium and potassium respectively are: injection, 0.3 per cent each; differential counting of serum (case A, Table II), 1.5 per cent and 1.4 per cent; counting of standards, 0.5 per cent each; and flame photometer, 0.5 per cent and 1.0 per cent. The accumulated effects of these errors on the values of the exchangeable body sodium and potassium are 1.7 per cent and 1.8 per cent, respectively.

RESULTS AND DISCUSSION

In order to establish the basic validity of the present differential counting technique for measuring the separate activities of sodium 24 and potassium 42 in mixtures of these isotopes, an *in vitro* trial of the method was first made. In these experiments known activities of Na^{24} and K^{42} in

three different mixtures (1:1, 3:1, 25:3 K^{42}/Na^{24}) of these isotopes were compared with the values derived by use of equations *5* and *6*. This comparison is presented in Table I from which it is apparent that the derived activities fall well within the limits of the counting errors of the corresponding known activities.

After satisfactory accuracy had been demonstrated *in vitro*, an *in vivo* study was undertaken. Separate determinations and a simultaneous determination of exchangeable body sodium and potassium were made on each of seven normal, adult males at one week intervals. The subjects were assumed to be in electrolyte balance during the experimental period. A comparison of the single measurements with their counterparts in the combined determinations is shown in Table III and demonstrates a satisfactory degree of reproducibility. No systematic error is evident in the signs of the differences. The coefficients of variation of the exchangeable body sodium and potassium measurements estimated from these seven cases are 3.2 per cent and 3.6 per cent, respectively. Thus differences of 9.0 per cent and 10.0 per cent, respectively, must exist between successive body sodium and potassium measurements to indicate a significant difference in these values by the criterion previously used (1).[10]

The coefficients of variation of the present method are somewhat higher than those obtained from similar measurements on a set of six subjects by the ion-exchange chromatographic method (1), 2.6 per cent for sodium and 2.0 per cent for potassium. The sodium values of 3.2 per cent for the present method and 2.6 per cent for the previous method are in good agreement.[11] The potassium values, 3.6 per cent and 2.0 per cent differ appreciably but they are not found to be significantly different on a 5 per cent probability basis. The discrepancy between the estimated total errors of the technique, 1.7 per cent for sodium and 1.8 per cent for potassium, and the coefficients of variation found from the *in vivo* data, 3.2 and 3.6 per cent, respectively, indicate the presence of other sources of variation, probably both technical and biological, which were not taken into account.

In addition to the simultaneous body sodium and potassium measurements in normal subjects, similar determinations have also been carried out on more than thirty patients with various metabolic abnormalities. The introduction of a differential counting technique has considerably simplified and facilitated simultaneous measurements of body electrolytes as compared with the procedures previously described (1, 10).

[10] Significance on a 2 S.D. or 4.6 per cent level of probability. The difference must exceed 2.83 × C. of V. for single determinations.

[11] Relative to the degrees of approximation in the estimates of the coefficients of variation based on six or seven cases.

SUMMARY

An improved method for the simultaneous determination of exchangeable body sodium and potassium, utilizing a differential counting technique is described. This technique makes it possible to measure the individual radioactivities of Na^{24} and K^{42} in solutions containing these isotopes without first separating them, as was heretofore necessary. The accuracy of the measurement of exchangeable body sodium and potassium by this method has been demonstrated by the satisfactory agreement obtained between single and simultaneously determined values on each of seven normal, male subjects.

The present procedure has been found to be considerably less complex and time-consuming than those previously used.

ACKNOWLEDGMENTS

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THE ORIGIN OF URINARY CREATINE IN PROGRESSIVE MUSCULAR DYSTROPHY [1]

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In an earlier communication (1) an experiment was described in which the isotopic compositions of urinary creatine and creatinine excreted by a patient with progressive muscular dystrophy were studied after a feeding of glycine-N^{15}. It was found, that in the first few days urinary creatine was much more enriched with N^{15} than was urinary creatinine, and that the isotope concentration in the creatine fell far more rapidly than did that in the creatinine. These results were taken to mean that the creatinuria, in this patient, was not a consequence of leakage of creatine out of skeletal muscle but rather represented newly formed creatine, synthesized in liver, which had failed to gain entry into muscle.

The present study represents an extension of these observations on two additional patients. Essentially the same technique as previously described was utilized. In view of the fact that the "sarcosine-nitrogen" of creatine derives from glycine nitrogen in liver, as does the nitrogen of hippuric acid, it was considered of interest to compare the abundance of N^{15} in simultaneously excreted creatine and hippurate after feeding glycine-N^{15}. To facilitate hippurate isolation from urine, small amounts of sodium benzoate were administered from time to time to each subject.

EXPERIMENTAL

Clinical histories

Subject T. K., a 31-year-old white male, has exhibited evidences of muscle dysfunction since the age of 4 and was diagnosed as having progressive muscular dystrophy at the age of 13. He has been unable to walk for the past 15 years. Marked wasting of the muscles of the shoulders, upper arms, spine, and thighs is apparent with some pseudohypertrophy of forearms, calves, and gluteal muscles. There was no evidence of disease of the nervous system.

Subject S. I. is a 28-year-old white male who has suffered from progressive muscular weakness since the age of 8. He has been unable to walk since the age of 16. Severe atrophy is noted in muscles of neck, upper arms, forearms, glutei, and thighs. He was considered a classical example of advanced progressive muscular dystrophy.

Experimental procedure

The subjects were maintained on a creatine-free, purine-poor diet throughout the experimental period. After four or five days, glycine-N^{15} was fed at 9 A.M. The dosage, as in the earlier experiments, was 100 mg. glycine per kilogram of body weight ($N^{15} = 60.2$ atom per cent excess). Each patient was continued on the diet for 14 days, and total urine was collected in 24-hour samples.

During this period, subject T. K. excreted an average of 320 mg. of creatine and 114 mg. of creatinine daily. Subject S. I. showed an average daily excretion of 685 mg. of creatine and 189 mg. of creatinine.

In the experiment on T. K. sodium benzoate, 450 mg., was injected intravenously immediately after breakfast on the 1st, 2nd, 4th and 8th day. Subject S. I. was given intravenous sodium benzoate two hours before breakfast on days 1, 2, 4 and 8. On day 9 he received sodium benzoate after breakfast to check the influence of a meal on the excretion of N^{15}. No essential difference was noted. The urine excreted during the two hours following each injection of sodium benzoate was separately collected, acidified to pH 3 with HCl and continuously extracted with ether for 12 hours. The ethereal extract was evaporated and the residue, after treatment with activated charcoal, was recrystallized from hot water. The melting points of the hippuric acid ranged between 187 to 189°.

Synthesis, isolations and degradations

Glycine-N^{15} was prepared essentially as described by Schoenheimer and Ratner (2) as modified by Muller and Bauer (3).

Each 24-hour urine sample was acidified with HCl to pH 3 or 4 and was then mechanically stirred with 30 gm.

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of Lloyd's reagent for three hours. The supernatant fluid, after centrifugation, was set aside. The solid phase was washed once with 0.1 N HCl. A preliminary study had revealed that essentially quantitative separation of creatine from creatinine was effected by this procedure, the creatinine being absorbed and the creatine remaining in the supernatant fluid. Creatinine was recovered from Lloyd's reagent by repeated elution with saturated aqueous $Ba(OH)_2$ solution until the eluate failed to give a Jaffe reaction. After treatment with CO_2 and removal of $BaCO_3$, the solution was evaporated under reduced pressure to about 10 ml., acidified to Congo Red paper with acetic acid and filtered if necessary. Creatinine was determined colorimetrically and one equivalent each of picric acid and potassium picrate were added in aqueous solution. The precipitate which formed over night was collected and recrystallized, as suggested by Bloch and Schoenheimer (4), from 0.12 per cent aqueous picric acid.

$C_4H_7ON_3 + C_6H_3O_7N_3 + KC_6H_2O_7N_7$

Calculated, creatinine 18.3

Found, creatinine 18.5

A weighed sample of potassium creatinine picrate was suspended in aqueous acid and exhaustively extracted with ether. A Kjeldahl nitrogen determination was conducted upon the colorless aqueous phase.

$C_4H_7ON_3 + C_6H_3O_7N_3 + KC_6H_2O_7N_7$

Calculated, creatinine N 6.9

Found, creatinine N 6.9

The supernatant solution from the treatment with Lloyd's reagent was treated with 42 ml. of conc. HCl per liter and the mixture was autoclaved at 15 lb. pressure for one hour, to convert creatine into creatinine. The latter product was then isolated as its potassium picrate double salt by the same procedure as is described above.

A trial separation was carried out by the foregoing procedures upon a synthetic mixture of creatinine (N^{15} = 0.073 atom per cent excess) and creatine ($N^{15} = 0$ atom per cent excess). Analyses of the separated components revealed no detectable isotopic enrichment in the creatine component, whereas the creatinine component contained 0.073 atom per cent excess.

Approximately 20 mg. of each sample of double salt were suspended in 0.1 N HCl and continuously extracted with boiling ether. Ether extraction was continued for at least one-half hour after the disappearance of yellow color from the aqueous phase. The colorless aqueous solution of creatinine hydrochloride was then digested by the Kjeldahl procedure in order to prepare the samples for mass spectrometric analyses of N.

The distribution of isotope in creatine was studied by the alkaline degradation of creatinine samples derived from urinary creatine. About 30 mg. of picrate double salt were heated under reflux in about 70 ml. of saturated aqueous $Ba(OH)_2$ solution. A continuous stream of N_2 swept the ammonia, formed from the amidine group of creatinine, into a receiver containing 5 ml. of 4 per cent boric acid solution (5). N^{15} abundance was determined in this ammonia, and from this value and the corresponding analysis of the creatine, the abundance of N^{15} in the sarcosine moiety was calculated from the equation:

$$N_S = 3N_C - 2N_A$$

where

N_S = atom per cent excess N^{15} in sarcosine moiety
N_C = atom per cent excess N^{15} in creatine
N_A = atom per cent excess N^{15} in ammonia liberated.

Analyses for creatine and creatinine were conducted by a modified Folin-Wu (6) method.

RESULTS AND DISCUSSION

The present experiments were predicated upon the assumption that if creatine and creatinine of the urine both arose from the same muscle creatine, they would at all times exhibit the same isotope enrichment. In our earlier study, following the feeding of glycine-N^{15} to a patient with progressive muscular dystrophy, this was found not to be the case. The results obtained in the present study (Figures 1 and 2) are in good qualitative agreement with the values previously published. Initially, far higher concentrations of N^{15} are found in urinary creatine than in urinary creatinine. The isotope concentration in creatine falls rapidly and by the 8th or 10th day is not sensibly different from that in the creatinine.

Creatinine is generally believed to arise in skeletal muscle from muscle creatine or phosphocrea-

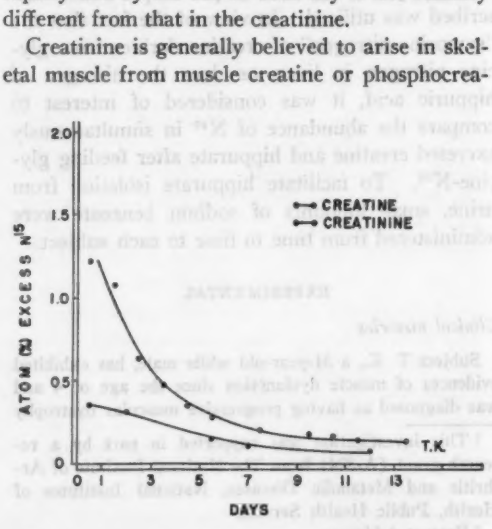

FIG. 1. ATOM PER CENT EXCESS N^{15} IN URINARY CREATINE AND CREATININE IN A PATIENT WITH PROGRESSIVE MUSCULAR DYSTROPHY AFTER INGESTION OF GLYCINE-N^{15}

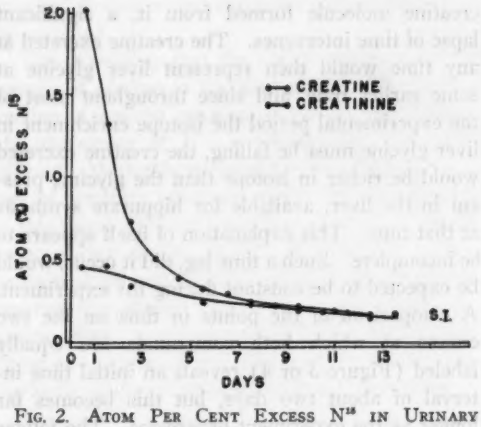

FIG. 2. ATOM PER CENT EXCESS N^{15} IN URINARY CREATINE AND CREATININE IN A PATIENT WITH PROGRESSIVE MUSCULAR DYSTROPHY AFTER INGESTION OF GLYCINE-N^{15}

tine (7). Isotope studies in animals (4, 8) have revealed that after suitable labeling procedures, the label is present in urinary creatinine at the same concentration as occurs simultaneously in muscle creatine, and this leads to the inference that muscle creatine is the unique precursor of urinary creatinine.

The present findings are taken to confirm our earlier conclusion that the urinary creatine in this disease did not leak out of muscle. From its initial high isotopic enrichment and from the subsequent rapid decline in abundance of N^{15} it is concluded that the creatine of the urine, in this disease, is freshly synthesized creatine which has never been mixed with the large stores of creatine contained in skeletal muscle. In man there is reason to believe that most or all creatine synthesis occurs in the liver (9) and it is, therefore, suggested that in progressive muscular dystrophy the creatinuria is a consequence of an imbalance between normal hepatic creatine synthesis, and markedly diminished creatine uptake by muscle. This view is in accord with that expressed by Hoagland, Gilder, and Shank (10).

The isotope concentrations observed in urinary creatinine in these two patients as well as in the patient studied previously, are higher than the corresponding values obtained from normal subjects (1). This suggests that, despite the great diminution in functional muscle mass, such muscle as survives is still very competent to convert creatine into creatinine. In fact the fraction of muscle creatine converted to creatinine daily is, if anything, greater than normal, although the absolute amount of creatinine formed is obviously less than normal.

It has been clearly established that the synthesis of creatine in animals involves the amidination of glycine at the expense of arginine followed by methylation of glycocyamine, at the expense of methionine. Glycine has been shown to be a specific biological donor of nitrogen to the sarcosine fragment obtained by alkaline degradation of creatine (11). Since this utilization of glycine in man is supposed to be largely a liver function and since hippurate formation is employed clinically as a measure of liver function, it was considered of interest to compare, after glycine-N^{15} ingestion, the concentrations of N^{15} in simultaneously excreted creatine and hippurate samples. The creatine values have been corrected for isotope contained in the amidine portion of the molecule (Tables I and II) and the results have been plotted on semilogarithmic coordinates, together with the hippurate data, in Figures 3 and 4.

Several points of interest emerge from these curves. In the first place, at all points in time in both subjects, there is a higher concentration of isotope in the sarcosine moiety of creatine than in the hippurate. Furthermore, the two curves, in each experiment, are roughly parallel on the coordinates employed, and indicate a fairly uniform ratio of isotope concentrations in the two products. The concentration of isotope in the sarcosine is

TABLE I

*Abundance and distribution of N^{15} in urinary creatine —Subject T. K.**

Day	Creatine	Amidine	Sarcosine
1.5	1.070	0.558	2.096
2.5	0.634	0.348	1.206
3.5	0.475	0.251	0.921
4.5	0.352	0.180	0.695
5.5	0.281	0.142	0.558
7.5	0.200	0.102	0.397
9.5	0.176	0.088	0.352
10.5	0.170	0.089	0.333
11.5	0.154	0.076	0.308
13.5	0.141	0.066	0.291

* From the concentrations of N^{15} in creatine samples and in the amidine nitrogen liberated from these samples, the concentrations of N^{15} in the sarcosine moiety have been calculated.

TABLE II

*Abundance and distribution of N^{15} in urinary creatine —Subject S. I.**

Day	Creatine	Amidine	Sarcosine
0.5	2.021	0.452	5.159
1.5	1.322	0.443	3.080
2.5	0.730	0.179	1.833
4.5	0.390	0.133	0.905
5.5	0 326	0.102	0.774
7.5	0.293	0.110	0.529
8.5	0.250	0.081	0.482
9.5	0.218	0.075	0.502
10.5	0.194	0.062	0.459
12.5	0.167	0.079	0.345
13.5	0.163	0.075	0.338

*From the concentrations of N^{15} in creatine samples and in the amidine nitrogen liberated from these samples, the concentrations of N^{15} in the sarcosine moiety have been calculated.

two to three times as high as in the hippuric acid. An additional point worthy of note is that the decline in isotope concentration is in no case a logarithmic function of time, indicating that this is not a simple first order process.

It had been anticipated that insofar as creatine synthesis and hippurate synthesis both tapped the glycine reserve of the liver, both products might show the same isotope concentrations in the present experiment. Two explanations may be invoked to account for the present finding of consistently greater enrichment of the sarcosine portion of creatine. The first of these relates to the possibility that between the moment of removal of a glycine molecule and the final excretion of the creatine molecule formed from it, a significant lapse of time intervenes. The creatine excreted at any time would then represent liver glycine at some earlier time, and since throughout most of the experimental period the isotope enrichment in liver glycine must be falling, the creatine excreted would be richer in isotope than the glycine, present in the liver, available for hippurate synthesis at that time. This explanation of itself appears to be incomplete. Such a time lag, did it occur, would be expected to be constant during the experiment. A comparison of the points in time on the two curves at which both compounds are equally labeled (Figure 3 or 4) reveals an initial time interval of about two days, but this becomes far longer as the experiment progresses. The failure

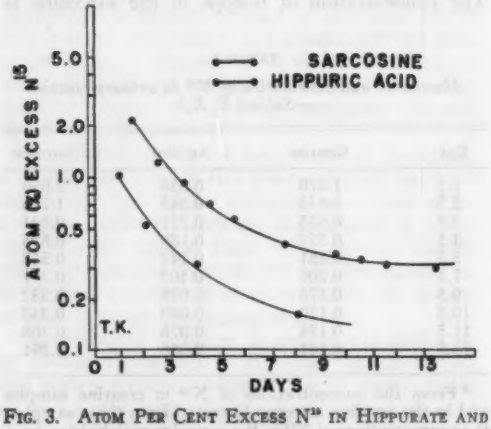

FIG. 3. ATOM PER CENT EXCESS N^{15} IN HIPPURATE AND IN SARCOSINE MOIETY OF CREATINE

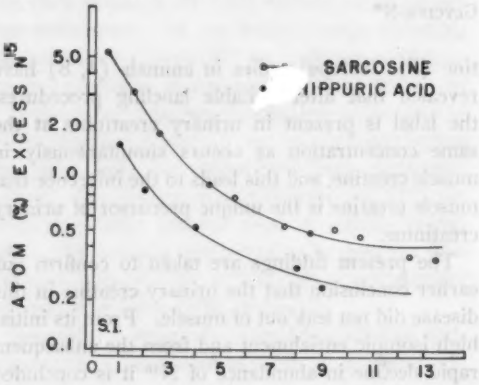

FIG. 4. ATOM PER CENT EXCESS N^{15} IN HIPPURATE AND IN SARCOSINE MOIETY OF CREATINE

to detect, in either experiment the ascending limb of the curve of N^{15} in sarcosine also argues against the occurrence of any significant time lag in creatine synthesis or in creatine excretion.

An alternative explanation is that there are in the liver at least two pools of glycine nitrogen which are not perfectly mixed with each other. Various mechanisms may be envisioned to account for the greater dilution of N^{15} in one pool than in the other, but at present information is insufficient to warrant speculation. The suggestion inherent in these results that isotope may be far from uniform in its distribution in one compound in one organ interjects a note of caution into interpretations of results of isotope experiments

in which homogeneous distribution of isotope must be assumed.

The possibility that the abrupt drain upon the supply of glycine in the liver imposed by the injection of sodium benzoate might of itself reduce the abundance of N^{15} in glycine has been considered. With this in mind we have kept the dosage of benzoate relatively small. However, even if this small additional drain of glycine did result in an abrupt decline in N^{15} concentration in that portion of glycine in liver used for hippurate formation, this change was not reflected in the sarcosine data. Consequently the conclusion that these two glycine derivatives tap discrete and imperfectly mixed reservoirs appears inescapable.

SUMMARY

Two patients with severe progressive muscular dystrophy have been fed glycine-N^{15} while on a creatine-free diet. The concentrations of N^{15} in urinary creatine and creatinine have been measured and compared. Initially far higher concentrations of isotope were found in creatine than in creatinine, and the decline in isotope abundance with time was more rapid in creatine than in creatinine.

These findings indicate that urinary creatine, in this disease, does not originate in muscle but represents freshly synthesized creatine which has been denied access to muscle.

A comparison of N^{15} concentrations in simultaneously excreted creatine and hippuric acid samples revealed that, at all points in time studied, the concentration of isotope in the sarcosine moiety of creatine exceeded that in hippuric acid by a factor of two- to three-fold. This finding has been taken as evidence of inhomogeneity in the glycine of liver available for synthetic processes.

ACKNOWLEDGMENT

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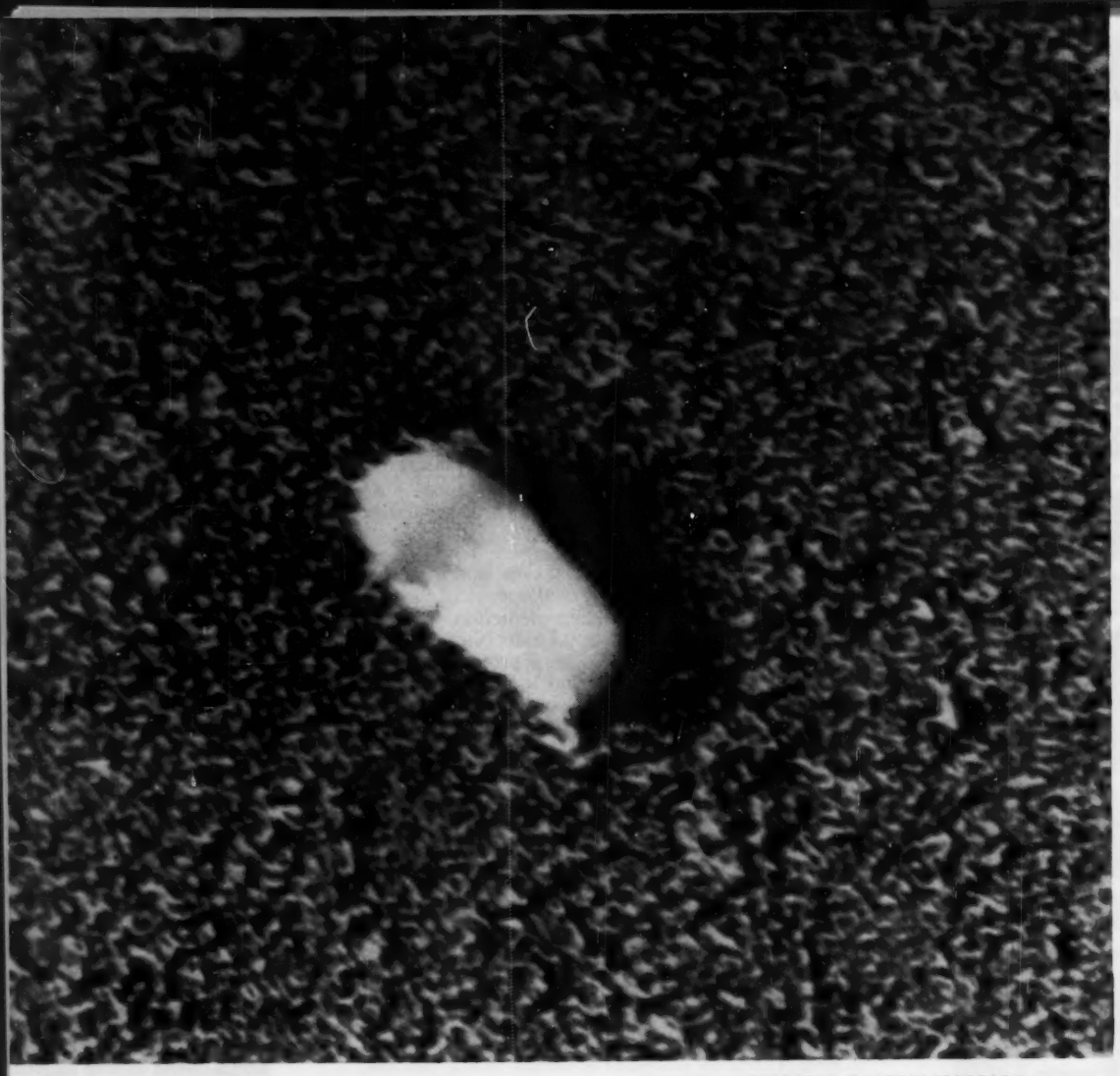

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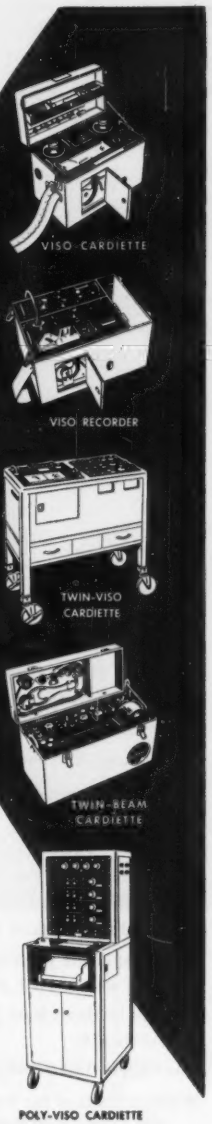

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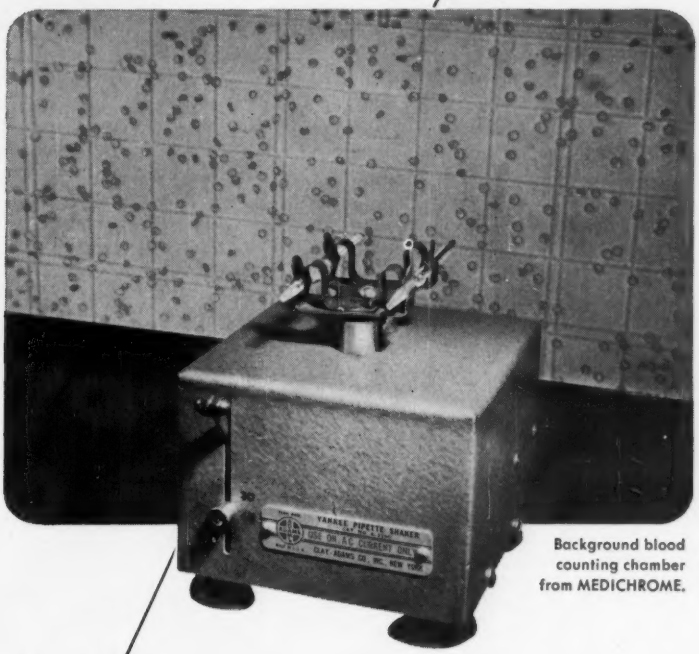

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